Phenotypic alterations and survival of monocytes following infection by human herpesvirus-6

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Summary. Freshly isolated monocytes rapidly undergo physiological changes in vitro, resulting in programmed cell death (apoptosis). Activation of monocytes, which promotes differentiation into macrophages, is known to inhibit apoptotic processes. In the present study, we report that human herpesvirus-6 (HHV-6) prevents monocytes from undergoing spontaneous apoptosis during the first 72 hours of culture. Furthermore, significant alterations in cell-surface phenotype were observed after 72 hours of infection with HHV-6. HHV-6-infected monocyte cultures have considerably reduced levels of CD14, CD64 (FcγRI) and HLA-DR antigen on their surface, while CD32 (FcγRII) expression is unaffected. On the basis of these results, we hypothesize that HHV-6 promotes monocytes survival and causes phenotypic modifications that could favor immune evasion and ensure its persistence within the infected host.

Introduction

Human herpesvirus 6 (HHV-6) was first isolated in 1986 from the peripheral blood of patients with AIDS or with lymphoproliferative disorders [28]. HHV-6 was identified as the etiologic agent of exanthem subitum, a common childhood illness characterized by high fever and skin rash [31]. A role for HHV-6 in diseases such as organ graft rejection [6], multiple sclerosis [2, 30] and AIDS (reviewed in Ref. [25]) has also been suggested. One particular aspect of HHV-6 pathogenesis is its ability to infect cells of hematopoietic origin. HHV-6 can infect T cells [21], B cells [28], NK cells [23], megakaryocytes [1] and monocytes/macrophages [16, 17]. It is also suggested that HHV-6 can establish a latent infection in the monocyte/macrophage lineage. During acute HHV-6 infection, the virus can be isolated from monocytes and T cells [18], while during the convalescent phase, HHV-6 could only be recovered from monocytes,
suggesting that these phagocytes can serve as reservoir for lifelong persistence of HHV-6.

Monocytes and macrophages are key effector cells of the immune system, engaged in host defense against infectious microorganisms. Under physiological conditions, monocytes circulate for 3 to 4 days in the human blood and then emigrate into the tissue where they either differentiate into macrophages or undergo apoptosis. The main functions of macrophages are to phagocytose and destroy particulate material and to present, in association with MHC II, processed antigens to T helper cells. Macrophages have a variety of surface receptors that bind particulate antigens such as bacteria; these include receptors for certain sugars like mannose and for lipopolysaccharide. Macrophages also express Fc receptors and complement receptors, enabling them to bind antigens that are coated with IgG antibodies and C3b complement protein, respectively.

Apoptosis is an energy-dependent process of cell suicide in response to a variety of stimuli. Apoptosis is genetically controlled and plays a protective role, eliminating cells that might prove harmful if they were to survive, and could help protect against virus infection by limiting viral spread. Several viruses affect apoptosis by interacting directly with components of the highly conserved biochemical pathway that regulates cell death. Many viruses block apoptosis to prevent premature death of the host cell to maximize virus progeny during lytic infection or to facilitate their persistence. Since viruses are obligate intracellular parasites, they must avoid premature cell death in order to complete their replication cycle.

In an effort to better understand HHV-6 survival and persistence in monocytes, we examined early infection events leading to the alteration of monocyte functions and allowing virus to escape immunosurveillance.

**Material and methods**

*Cell culture and reagents*

Peripheral blood mononuclear cells (PBMC) were obtained from venous blood of healthy donors and isolated by centrifugation over lymphocyte separation medium. Monocytes were first enriched by centrifugation over a Percoll density gradient as described [4]. Monocytes were further enriched by plating mononuclear cells (12 × 10^6 cells/well) in 6-well tissue culture plates (Costar, Cambridge, MA) and incubated for 2 h at 37 °C in an atmosphere of 5% CO₂. Non-adherent cells were then removed and the adherent monolayers were washed several times with PBS. This procedure yielded >80% pure monocytes as determined by side scatter and CD14 expression. As anticipated, the plasma of all blood donors were positive for HHV-6 antibodies, as determined by standard immunofluorescence on HHV-6 infected cells, indicating prior infection by this virus. However, no signs of active infection (HHV-6 in plasma) were noted, as determined by TAQman PCR. Furthermore, in the absence of exogenously added virus, we could not detect HHV-6 DNA in freshly isolated or cultured monocytes.

*Viral preparation*

The HHV-6 (Variant B, Z29 strain) used in this study was propagated on Molt-3 cells as described [9]. After 7 days of infection, virus was concentrated from culture supernatant by
centrifugation (38,800 x g, 2 h 40 min) and resuspended in a minimal volume of complete culture medium. HHV-6 infectivity titer was determined on MOLT-3 cells, 24 h after infection with varying dilutions of HHV-6. Infected cells were fixed in cold acetone for 10 min, air-dried, and reacted with Alexa 488-labeled anti-IE1 antibodies for 1 h at room temperature. After three 5-min washes in PBS, slides were mounted with glycerol and observed under fluorescent microscopy. After calculating the percentage of IE1-positive cells, the HHV-6 titer was determined to be $6 \times 10^6$ infectious particles/ml.

_Infection procedure_

Monocytes (10^5 cells/well) were incubated with infectious HHV-6 or UV-irradiated HHV-6 (1.2 J/cm^2) at a multiplicity of 1 in 1 ml of culture medium for 4 h at 37°C and were washed twice with PBS. Cells were subsequently cultured in 1 ml of culture medium and cultured for 72 h. Effectiveness of UV-irradiation was determined by confirming the lack of IE1 expression (assayed by IFA), following a two-day exposure of MOLT-3 cells to inactivated viral preparation. In some experiments, monocytes were treated (100 µg/ml) with the viral DNA polymerase inhibitor phosphonoacetic acid (PAA) prior to and during the course of HHV-6 infection. Effectiveness of PAA was previously confirmed in our laboratory by the ability of PAA to restrict HHV-6 gene expression to IE and early (E) genes while preventing viral DNA replication. These assays were performed by IFA using anti-IE1, anti-p41 antibodies as well as DNA slot blot hybridization to monitor HHV-6 viral DNA.

_mRNA analysis_

Total RNA was isolated from mock- or HHV-6-infected monocytes using the TRIzol reagent (Invitrogen). Total RNA was treated with 2 units of RNase-free DNase for 30 min, phenol-extracted, and ethanol-precipitated. 5 µg of total RNA was separated by agarose gel electrophoresis and transferred by capillary diffusion onto a nylon membrane. RNA was cross-linked to membrane by UV exposure (1200 J). The membrane was incubated at 42°C in a prehybridization buffer (50% formamide, 10% dextran sulfate, 2X SSC, 1% SDS, and 1X Denhardt’s solution) for 2 h. Hybridization was performed overnight in the same buffer containing $1 \times 10^6$ cpm/ml of the 32P-labeled oligonucleotide probe specific for CD14. The membrane was subsequently washed once in 2X SSPE for 15 min at 42°C, twice with 2X SSPE, 2% SDS for an additional 30 min at 42°C, and once with 0.1X SSPE at 42°C for 15 min before being exposed to film. Hybridization with probe specific for the 18S ribosomal subunit was used as an internal control to assess the quantity of RNA loaded.

_Flow cytometry analysis_

Anti-CD14 and isotype IgG2a FITC or PE-labeled antibodies, anti-CD32, and anti-CD64 were all obtained from BD Biosciences (Mississauga, ON); the secondary antibody was an FITC-labeled goat anti-mouse IgG from BD Biosciences (Mississauga, ON). Cells treated with antibodies were incubated for 30 min on ice and then washed twice with saline. The cells stained by the indirect method were resuspended in the secondary antibody at a 1:100 dilution for 30 min and then washed twice prior to flow cytometry analyses (Beckman Coulter, Miami, Fl). Mean fluorescence intensity and percentages of positive and negative cells were analyzed by gating on the monocyte population defined by forward and side light scatter. As a negative control, cells were incubated with appropriate isotype-matched control antibodies. Ten thousand events were recorded for each sample and the results are expressed as mean fluorescence intensity (MFI).
Determination of apoptosis

Cells (10^5) were washed with PBS and resuspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl_2). FITC-Annexin V (R&D Systems, Minneapolis, MN) and PE-CD14 (BD Biosciences, Mississauga, ON) were added according to the manufacturer’s instructions. Labeled cells were analyzed by flow cytometry.

Results

**HHV-6 infection prevents monocytes from undergoing spontaneous apoptosis**

From our initial observations that considerably higher numbers of damaged and/or dead monocytes were present in uninfected cultures compared to that of HHV-6-infected cultures, we studied whether this virus could influence monocytes survival. The first notable observation made relates to the difference in monocyte morphology between uninfected and HHV-6-infected cultures (72 h). As shown (Fig. 1A) in the side scatter/forward scatter plots, a large proportion of cells (>50%) from uninfected cultures have reduced size, reminiscent of cells in the process of dying. This is in contrast to HHV-6-infected cultures, where most of the cells have normal-appearing size and morphology. To assess the proportion

![Fig. 1](image_url)

Fig. 1. HHV-6 infection prevents monocytes from undergoing spontaneous apoptosis. Monocytes were cultured for 72 h in the presence or in the absence of HHV-6. A Side and forward scatters of HHV-6-infected and mock-infected monocytes. B Cells were stained with FITC-labeled Annexin-V to detect early apoptotic cells. The figure shown is representative of three independent experiments.
of apoptotic cells, monocytes were cultured in the absence or in the presence of HHV-6 for 72 h and subsequently labeled with FITC-annexin-V. The detection of phosphatidylserine exposure through annexin-V binding is considered a general

![Histograms showing CD14 and annexin-V expression](image)

**Fig. 2.** Decreased surface expression of CD14 on annexin-V− monocytes from HHV-6 infected culture. A Monocytes were cultured for 72 h in the presence or in the absence of HHV-6 and analysed for CD14 and annexin-V expression. The percentage of cells in each quadrant is presented in the top left corner of each histogram. The mean fluorescence intensity (MFI) of CD14+/Annexin-V− (lower right quadrant) is also presented below the panels. B Surface expression (MFI) of CD14 on mock (NI) or HHV-6-infected, Annexin-V− monocytes incubated in the absence or in the presence of PAA (100 µg/ml) or with UV-irradiated (1.2 J/cm²) HHV-6. Results are presented as mean CD14 MFI ± S.D. on Annexin-V− monocytes from three independent experiments. C Northern blot analysis of CD14 mRNA in human monocytes incubated in the presence or in the absence of HHV-6. Each lane was loaded with 5 µg of total RNA. The blot was hybridized with 32P-labeled CD14 probe. Blots were also stripped and rehybridized with 32P-labeled 18S ribosomal subunit probe showing equal RNA loading. The figure shown is representative of three independent experiments.
and early marker of programmed cell death [19]. As shown in Fig. 1B, 35% of cells from the uninfected monocyte cultures stained with annexin-V, suggesting that these cells are in the process of dying by apoptosis. In contrast, only 3% of cells from HHV-6-infected monocyte cultures reacted with annexin-V.

**Decreased surface expression of CD14 on monocytes from HHV-6-infected culture**

From the results presented in Fig. 1, it is clear that HHV-6 infection is associated with monocyte survival. To better characterize these observations, uninfected and HHV-6-infected monocyte cultures were dually-labeled with annexin-V and CD14, a cell surface monocyte marker. As presented in Fig. 2A, the percentage of annexin-V positive cells in uninfected cultures was 10 times higher (56% versus 5%) than for cells from HHV-6-infected cultures. Furthermore, annexin-V-positive cells had much reduced levels of CD14 compared to annexin-V-negative ones, irrespective of whether they were infected or not. This is not uncommon as dying cells were reported to have a tendency to lose CD14 expression (see discussion). The interesting observation to be made concerns CD14 expression on annexin-V-negative cells. When analysis is performed by gating on CD14^+^-Annexin-V^- cells (bottom right quadrants of histograms in Fig. 2A), an important reduction in CD14 expression at the surface of monocytes from HHV-6-infected cultures is observed when compared to mock-infected ones. By measuring CD14 expression through the mean fluorescence intensity (MFI), this translates into a 3-fold reduction (315 versus 98) in CD14 expression.

To determine if CD14 reduction following HHV-6 infection required active viral replication, HHV-6 was inactivated by UV irradiation. UV irradiation causes DNA damage and prevents viral gene transcription with minimal effects on viral particle structural integrity and entry processes. When Annexin-V^-/CD14^+^ cells were studied, wild-type infectious HHV-6 caused a 3-fold reduction in CD14 expression (Fig. 2B). In contrast, UV-irradiated HHV-6 proved incapable of reducing CD14 expression levels, suggesting that binding to the cell surface and entry of virus into the monocytes are not sufficient to cause CD14 downregulation. However, monocytes incubated with UV-inactivated HHV-6 did not undergo apoptosis, suggesting that CD14 downmodulation and monocyte survival are caused by two different mechanisms. From our results we conclude that viral gene transcription is needed for CD14 downmodulation while it is not essential for HHV-6 to promote monocyte survival. In an attempt to determine whether early or late HHV-6 proteins are playing a role in the noticed CD14 downregulation, we performed experiments in the presence of PAA, a viral DNA polymerase inhibitor that allows expression of immediate-early and early HHV-6 proteins while preventing late protein synthesis [5]. In the presence of PAA, HHV-6 retained its ability to decrease CD14 expression on the surface of human monocytes (Fig. 2B), suggesting that immediate-early or early HHV-6 proteins are likely responsible for this effect. Lastly, to assess whether CD14 inhibition of expression by HHV-6 is associated with a decrease in CD14 mRNA, Northern analysis was performed. Monocytes
Fig. 3. HHV-6 reduces HLA-DR, FCγRI but not FCγRII surface expression on human monocytes. Human monocytes were incubated in the presence or in the absence of HHV-6 for 72 h. Cells were then labeled and analyzed for A HLA-DR, B CD14/CD64 or CD14/CD32 expression by flow cytometry. The percentage of cells in each quadrant is presented in the top left corner of each histogram. The figure shown is representative of three independent experiments.
were cultured for 72 h in the presence or in the absence of HHV-6, and RNA was extracted from the entire cell populations (Annexin-V$^-$ and Annexin-V$^+$) and probed for CD14 mRNA expression. Figure 2C demonstrates that infected cells have much reduced levels of CD14 mRNA compared to mock-infected cells. This result suggests that many Annexin-V$^+$ cells are viewed as CD14$^-$ by flow cytometry, but are still expressing CD14 mRNA. This is not uncommon since numerous examples exist of reduction of cell surface marker expression without a noticeable effect on gene transcription (see discussion).

**HHV-6 reduces HLA-DR, CD64 (FC$_\gamma$R I) but not CD32 (FC$_\gamma$R II) surface expression on human monocytes**

To further characterize the phenotype of monocytes from HHV-6-infected cultures, cells were labeled with various monoclonal antibodies directed against cell-surface markers known to be important for monocyte functions. Following 72 hours of culture in the presence or in the absence of HHV-6, the cells were stained and analyzed by flow cytometry. As shown in Fig. 3A, HHV-6 downregulates surface expression of HLA-DR as compared with mock-infected cells. As estimated by the mean fluorescence intensity, HHV-6 caused a 2-fold reduction in HLA-DR expression (425 versus 222). Similar results were obtained when CD64 expression was studied (Fig. 3B middle panels). As for HLA-DR, HHV-6 infection caused a significant down-regulation of this marker with a 2-fold reduction in CD64 intensity. In contrast to HLA-DR, CD14, and CD64, the expression of the low-affinity FC$_\gamma$RII (CD32) remained unaffected (Fig. 3 bottom panels) by HHV-6, suggesting that this virus selectively represses cell-surface molecule expression.

**Discussion**

In the present study, we report that HHV-6 infection protects human monocytes from spontaneous apoptosis occurring during the first 3 days of culture. The mechanisms involved in the regulation of monocyte apoptosis are only partially understood. Several authors claim the existence of 2 distinct pathways; one by which resting monocytes enter into programmed cell death and another by which activated cells, undergoing differentiating processes, are protected from apoptosis [26, 27]. We also demonstrate that the expression of CD14 on annexin-V$^-$ human monocytes is downregulated following exposure to HHV-6 compared to that of live monocytes from uninfected cultures. HHV-6-induced downregulation of CD14 is associated with reduced CD14 mRNA, suggesting either an effect on transcription of the CD14 gene or a shortened half-life of the corresponding mRNA. The results in Fig. 2C, which show a majority of apoptotic cells from uninfected cultures having reduced CD14 surface expression, yet having a stronger CD14 mRNA hybridization signal compared to monocytes from HHV-6-infected cultures, can be explained as follows: first, apoptotic monocytes from uninfected cultures, for which CD14 cell surface expression was severely diminished, continued to express normal CD14 mRNA with possible inhibition of translation events often observed
during apoptosis. Alternatively, it is also known that degradation of cellular mRNA occurs early after apoptosis induction [3], suggesting that the only source of intact mRNA in uninfected cells would come from live monocytes which express high levels of CD14.

Several studies suggest that CD14 acts not only as a surface receptor for LPS but also functions as a polyspecific receptor with broad recognition properties. In fact, it is suggested that CD14 is involved in the regulation of monocyte apoptosis, considering that activating stimuli, which are able to enhance CD14 expression, protect monocytes from apoptosis. Heidenreich and al. reported that downregulation of CD14 expression represents a trigger for the induction of monocyte apoptosis [12]. Our results confirm that the loss of CD14 surface marker is accompanied by spontaneous apoptosis in uninfected cells. On the other hand, we also observed CD14 downregulation in HHV-6-infected monocyte culture that showed prolonged survival and no signs of apoptosis. Our data therefore suggest that lower (3-fold) than normal CD14 expression is compatible with cell survival. Attempts to detect HHV-6 antigens (IE1 and gp106) in monocytes proved unsuccessful. Immunofluorescence studies of primary monocytes proved very difficult to perform for technical reasons. We were therefore unable to assess the percentage of HHV-6-infected monocytes in our culture. As an alternative, real-time PCR experiments were conducted to determine the quantity of HHV-6 DNA in monocyte cultures 72 h after viral infection. Our results indicate that, on average, 21 copies of HHV-6 viral DNA were detected per monocyte. Whether this reflects a few cells carrying many (>21) copies of viral DNA or the majority of cells carrying 21 copies of the HHV-6 genome could not be determined. At present, we cannot confirm that the observed effects on cell surface markers are directly linked to HHV-6 infection. Whether other factors contribute to promote survival of monocytes in infected cultures remains to be determined. One factor previously reported to promote monocyte survival is TNF-α. Interestingly, a previous study has shown that HHV-6 is a potent inducer of TNF-α and that secretion of this cytokine occurs with kinetics compatible with a possible influence of this factor on monocyte survival [7]. However, experiments designed to neutralize endogenously secreted TNF-α in response to HHV-6 suggest that this cytokine has no impact on monocyte survival in this system (data not shown). Further studies are therefore needed to identify the factors contributing to monocyte survival following HHV-6 infection.

Besides survival and the downregulation of CD14, HHV-6-infected monocytes undergo changes in the expression of several other surface molecules. Unlike mock-infected cells, monocytes from HHV-6-infected cultures displayed a significant reduction in HLA-DR antigen expression, which could lead to a substantial loss in their antigen-presenting capacity to T cells. In fact, the major histocompatibility complex molecules constitute an interface between the host and the pathogen. MHC molecules present peptides to T lymphocytes, thereby triggering an immune response that is essential in controlling most viral infections. Not surprisingly, several viruses have evolved diverse strategies for inhibiting MHC molecule expression and thus escaping immune system surveillance. For example, several human herpesviruses such as human cytomegalovirus (CMV), Varicella
zoster virus (VZV), herpes simplex virus 1 (HSV-1), and Epstein-Barr virus (EBV) silence MHC class II molecule expression in a variety of cells (reviewed in [11]). It has also been reported that HHV-6 variant A but not HHV-6B downregulates MHC class I expression on dendritic cells [13]. In contrast, another study suggests that the variant B induces the expression of both MHC class I and class II molecules on these cells [15]. The mechanisms underlying the HLA-DR down-regulation on monocytes following HHV-6 infection need to be identified, and the true impact of such phenotypic alterations as an immune evasion strategy remains to be determined experimentally.

Interestingly, HHV-6 also down-regulates surface expression of FcγRI. These receptors, which bind the constant region of IgGs, are the major mechanism by which cells of monocyte lineage recognize IgG-opsonized pathogens, thereby triggering phagocytosis and Ab-dependent cellular cytotoxicity. Several viruses are able to overcome this arm of the immune system. Some viruses such as herpesviruses and coronaviruses produce molecules that bind to the Fc region of host immunoglobulins, preventing neutralization of free virus and engaging in antibody-dependent activity against infected cells [20]. HCMV and HSV induce Fc-binding activity in infected cells by encoding proteins with Fc-binding capabilities. Many viruses are not neutralized by antibodies and must be destroyed by other means. To dispose of neutralized virus, antibodies can bind to cells like monocytes bearing Fc receptors which ingest antibody-coated virus and destroy them. By downregulating FcγRI, HHV-6 may reduce phagocytosis of opsonized HHV-6 by monocyte and then silencing proinflammatory response of its reservoir cells in order to evade immunosurveillance. Previous studies have shown that HHV-6 infection can modulate the expression of several other receptors. The observation that human herpesvirus 6 (HHV-6) can induce CD4 gene transcription and expression in CD4(−) cells was reported several years ago [8, 22, 24]. The transcriptional down-regulation of the CD3/TCR complex, by affecting a critical T cell recognition function, could also be relevant to HHV-6 immunoevasion strategies [8].

The first genes expressed following viral entry and associated/responsible for many cellular disturbances are the immediate-early (IE) genes. Transcription of IE genes occurs within minutes to hours post-entry. The HHV-6 genome contains several putative IE genes including IE1 and IE2, both expressed during latency (H6LT) and the productive-phase (IE1 and IE2) [9, 10, 18, 29]. We previously reported that the U89 and U86 transcripts encoding IE1 and IE2, respectively, can be detected in HHV-6-infected monocytes [14]. Kondo et al. recently shown that it is possible to detect IE1 and IE2 transcript (H6LT) in latently-infected cells [18]. Thus, it is possible that these proteins play a role in phenotypic switching of HHV-6-infected monocytes, allowing the virus to establish a latent infection in these cells in order to escape immune system surveillance.

In order to survive in the presence of an active immune response, viruses have evolved several mechanisms to evade the host’s immune system. The silencing of proinflammatory function of monocytes/macrophages through the downregulation of HLA-DR, CD14, and CD64, which would delay the development of an
immune response, represents a potential survival strategy that allows HHV-6 to persist for long periods of time in monocytes/macrophages.

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