Understanding the Molecular Mechanisms Involved in Indirect Effects of Cytomegalovirus

Cecilia Söderberg-Nauclér, Mensur Dzabic and Afsar Rahbar

Department of Medicine, Center for Molecular Medicine, Karolinska Institute, Stockholm, Sweden

Abstract

Human cytomegalovirus causes clinical problems in organ transplant recipients through both direct and indirect effects. The direct effects are mediated by the destruction of infected cells by a lytic infection or by elimination of infected cells by the immune system. However, the mechanisms for the indirect effects are only beginning to be better understood. The best-studied indirect effects linked to cytomegalovirus infection include acute and chronic rejection, increased risk of bacterial and fungal infections, cardiovascular disease, and posttransplant diabetes. Prophylaxis against cytomegalovirus seems to lower the incidence of some of these complications, suggesting that the long-term effects are mediated by viral replication. However, in patients it has been difficult to detect the virus with conventional methods. To detect low-grade active cytomegalovirus infection in transplanted organs, we developed a high-sensitivity immunohistochemistry technique. Using this technique, we found cytomegalovirus in a majority of grafts with chronic rejection. Thus, active viral replication in these grafts might adversely affect their function. In infected cells, cytomegalovirus can produce over 250 proteins, but only 50-60 are essential for viral replication. Evidently, the vast majority of cytomegalovirus proteins arose to help the virus coexist in its host. Acting through sophisticated mechanisms, these proteins target many host cell functions, including cellular differentiation, cell-cycle regulation, DNA repair mechanisms, epigenetic functions, apoptosis, cellular migration, lipid metabolism, thrombogenesis, angiogenesis, and immune evasion, that may explain a majority of the indirect effects of cytomegalovirus. Here, we highlight the molecular mechanisms that may underlie the indirect effects of cytomegalovirus in transplant recipients and severely impair their long-term outcome. (Trends in Transplant. 2008;2:32-43)

Corresponding author: Cecilia Söderberg-Nauclér, cecilia.naucler@ki.se

Key words


Correspondence to:
Cecilia Söderberg-Nauclér
Center for Molecular Medicine, L8:03
Karolinska Institutet at Karolinska Hospital
SE-171 76 Stockholm
E-mail: cecilia.naucler@ki.se
Introduction

Human cytomegalovirus (CMV) was first described in 1898\(^1\), but was not defined as a virus until the 1950s. Initially, it was believed to cause only rare cases of CMV inclusion disease in infants with severe congenital infection. In the 1960s, CMV infection was reported in immunocompetent individuals as a posttransfusion syndrome similar to mononucleosis\(^2\). In the 1970s and 1980s, when large groups of patients became immunosuppressed as a result of organ transplantation and AIDS, CMV infection emerged as an important cause of morbidity and mortality. Knowledge about CMV expanded quickly, rapid diagnostic methods to detect the virus became available, and antiviral drugs that control CMV replication and disease progression became life saving for many patients. At that time, many clinicians felt that CMV was no longer a clinical problem in the management of these patients. However, even in the 1970s it was noted that CMV infection is associated with rejection. In 1983, Lönningquist, et al. reported that CMV infection increased the risk of chronic graft-versus-host disease in bone marrow transplant recipients\(^3\). In 1989, Grattan, et al. demonstrated that solid organ transplant recipients with CMV infection were at high risk of chronic rejection in heart grafts\(^4\).

Today, it is clear that patients with CMV infection are at increased risk for a number of long-term complications after transplantation\(^5,6\). These include acute and chronic rejection in the graft, (bronchiolitis obliterans, vanishing bile duct syndrome, transplant vascular sclerosis), increased risk of bacterial and fungal infections, cardiovascular disease, posttransplant diabetes, and malignancies (Fig. 1). These complications are considered to be indirect effects of CMV and reveal the potent effects of the virus, even though active viral infection has been difficult to detect in patients or targeted organs by conventional methods.

In solid organ transplant recipients, CMV is considered to cause both direct and indirect effects\(^7\). The direct effects reflect the destruction of virus-infected cells by a lytic infection or by the immune system. This scenario is exemplified by prolonged fever and leukopenia (CMV syndrome) and organ-invasive disease such as hepatitis, gastrointestinal disease, pneumonitis, pancreatitis, carditis, and retinitis (Fig. 1). Since CMV seropositivity and asymptomatic viremia also increase the risk for long-term complications in transplant patients, high viral titers may not be necessary to increase the risk of indirect effects of CMV. In seropositive individuals, the virus may be replicating and causing harm, even though an active infection cannot be detected by conventional methods. In support of this hypothesis, CMV proteins produce the most immunodominant peptides ever seen by our immune system\(^8\). Indeed, in healthy adult carriers of the virus, 30-50% of the T-cell repertoire may be specific for CMV. This finding is consistent with the notion that the immune system frequently recognizes CMV peptides, suggesting that CMV reactivation periodically occurs in immunocompetent individuals without clinical signs of infection. Furthermore, in transplant patients, prophylaxis against CMV reduces the risk of some of the indirect effects of the virus, providing additional support for the hypothesis that active viral replication is involved\(^9\).

Cytomegalovirus produces nonessential viral proteins with potent effects on host functions

Cytomegalovirus belongs to the β-herpesvirus family. Like other herpesviruses, it establishes latency and persists after a primary infection, and it cannot be cleared from the host by the immune system. The virus has
adapted to exist in immunocompetent individuals, reflecting tremendous evolutionary pressure both to develop strategies for avoiding detection and elimination by the immune system and to modify infected cells as efficient virus factories. In clinical isolates, 252 open reading frames have been identified in the viral genome, suggesting that CMV can produce over 250 proteins in infected cells. However, only 50-60 are considered essential for viral replication (i.e. to produce new viral progeny). The remaining 200 proteins act through specific and sophisticated mechanisms to control important cellular and immunologic functions, enabling the virus to coexist in the host. These proteins could also contribute to the development of many common diseases.\textsuperscript{10}
Cytomegalovirus is reactivated by inflammation

During latency, the virus appears to be silent and causes no clinical symptoms as long as it is kept in balance with the host immune system. The viral DNA in latently infected monocytes may remain in an extrachromosomal circular form, and few if any viral proteins are produced during the latency phase. Since immunosuppressed patients develop CMV infection and disease, it was early hypothesized that immunosuppression would lead to reactivation of latent virus. However, we showed that allogeneically stimulated T-cells produce inflammatory cytokines, such as tumor necrosis factor-α (TNFα) and interferon-γ (IFNγ) that cause monocytes to differentiate into inflammatory macrophages, which can reactivate latent CMV. This scenario would likely take place during episodes of acute rejection or acute graft-versus-host disease in recipients of organ and stem-cell transplants.

Owing to major histocompatibility complex (MHC) mismatch, virus-specific cytotoxic T-cells have a decreased ability to clear CMV infection from the graft. This is most likely why CMV infection is always far more problematic in transplanted organs than in native organs. Furthermore, immunosuppression impairs the ability to control the reactivated virus, placing patients at high risk for clinical CMV disease. The T-cell activation that induces inflammatory cytokine production probably occurs after other infections as well, which may explain why AIDS patients are at high risk for developing symptomatic CMV infection. The virus may also be reactivated in tissues affected by inflammation in patients with inflammatory diseases such as autoimmune disorders, cardiovascular diseases, and certain cancers. In such patients, reactivated CMV may spread locally to other cells and, through its unique ability to control host functions, may contribute to the clinical course of the disease. Cytomegalovirus is now being discovered in several of these diseases, but it is unknown whether the virus plays a causative role or is merely an epiphenomenon of inflammation.

Cytomegalovirus can not only be reactivated by inflammation, it also appears to be dependent on inflammation. As a result of its evolutionary adaptation to its host, the virus can both induce and enhance inflammation. It induces cyclooxygenase 2 (COX-2) expression, and its replication in vitro is attenuated by aspirin. In rat liver allografts, COX-2 expression is enhanced by CMV infection and acute rejection. Also, CMV induces expression of 5-lipoxygenase and production of leukotriene B4 in inflamed tissues and is associated with massive infiltration of inflammatory cells in patients with inflammatory diseases. Thus, CMV reactivation may lead to production of leukotriene B4 and downstream COX-2 metabolites that further enhance both viral replication and the recruitment of inflammatory cells into the tissue.

In support of this hypothesis, CMV-induced COX-2 expression increases viral replication via the prostaglandin E pathway in epithelial cells. This may enhance viral replication and lead to the accumulation of viral proteins that can be degraded and presented to T-cells, thereby further enhancing and sustaining local tissue inflammation. Furthermore, CMV-infected cells themselves exhibit enhanced production of transforming growth factor-β (TGFβ), TNFα, interleukin (IL)-1β, IL-6, IL-8, oncostatin M, platelet-derived growth factor, basic fibroblast growth factor, monocyte chemoattractant protein 1, and regulated upon activation, normal T-cell expressed, and secreted (RANTES), all of which may promote continued inflammation.

The product of the CMV immediate-early 1 product increases promoter activity in the IL-6 gene by binding to the nuclear
binding protein NFκB. The ability to induce IL-6 production may contribute to CMV-associated inflammation (reviewed10). Furthermore, the CMV genome encodes four homologs of chemokine receptors (US27, US28, UL33, UL78), as well as homologs of the TNFα receptor, IL-8, IL-10, and a human leukocyte antigen (HLA) class-I molecule that may modulate the immune responses of the host. In addition, UL146 and UL147 are similar in size and sequence to alpha (CXC) chemokines. The IL-8 enhances CMV replication in fibroblasts through interactions with IL-8 receptors, and CMV infection enhances IL-8 expression (reviewed10).

Cytomegalovirus infection and acute rejection in solid organ transplant recipients

Cytomegalovirus infection has long been associated with acute rejection episodes in organ transplant recipients20-25. Initially it was observed that clinical disease often was associated with acute rejection episodes, but it has been difficult to define whether CMV or rejection represents the egg or the chicken in this process. Clearly, a bidirectional interaction exists between the virus and acute rejection6, as exemplified by CMV’s ability to induce and be dependent on inflammation. In more recent studies, detection of CMV DNA by sensitive methods correlated with increased creatinine levels in kidney transplant recipients26. Thus, CMV may in fact be actively replicating in the kidney, although the virus could be discovered only by sensitive in situ hybridization and polymerase chain reaction techniques.

Recently, we adapted methods for sensitive detection of active CMV infection in tumors for use in tissues from organ transplant recipients. Using high-sensitivity immunohistochemistry staining (HSIS) protocols, we can now detect a low-grade active CMV replication in a very high proportion of solid organ transplants diagnosed with acute and chronic rejection (unpublished data). In heart transplant patients, we detected an active CMV infection in 20 of 20 patients (Fig. 2 and unpublished data). The virus appeared to be detected earlier than rejection, and the viral levels appeared to correlate with the rejection grade over time (Fig. 2). Thus, with these new techniques it may be possible to identify patients who are at high risk of rejection and who should be offered antiviral treatment. Indeed, aggressive prophylaxis against CMV in heart transplant patients reduces rejection8,25, whereas preemptive treatment against CMV prevents CMV disease but not acute rejection27.

Cytomegalovirus infection and chronic rejection

Since the first report that CMV infection increases the risk of transplant coronary artery disease in 19894, strong evidence has emerged that the virus contributes to chronic deterioration of transplanted organs. The virus is clearly associated with chronic rejection (i.e. transplant vasculopathy, chronic allograft nephropathy, bronchiolitis obliterans, vanishing bile duct syndrome, and fibrosis) in transplanted organs23,26,28-31. In animal models, CMV infection consistently induces earlier and more advanced lesions, which suggests that the virus is a strong cofactor in the development of these diseases (reviewed32). The effect of CMV is linked to rejection, and prophylaxis against CMV and optimal rejection treatment prevents CMV-induced graft damage (reviewed6).

Recently, using the HSIS technique, we examined kidney biopsies from 20 transplanted patients with chronic rejection. Active CMV micro-infection was detected in all 20 biopsies, but not in kidneys from CMV-
Seronegative subjects (Fig. 3 and unpublished data). Similar findings were obtained in preliminary studies of recipients of lung and liver transplants. Importantly, active viral infection is often detected in areas of the graft with clear disease pathology involving vascular changes and fibrosis. Since CMV has been strongly linked to chronic rejection, it is important to determine whether a low-grade CMV infection in the graft is a cause of rejection or simply an epiphenomenon of inflammation. Solid evidence from transplant patients favors the hypothesis that the virus causes or is a cofactor in many long-term complications, suggesting that it contributes to disease pathogenesis at the molecular level. However, it is extremely difficult to link a particular virus to a disease that may not produce clinical symptoms for several years. Therefore, in vitro models of isolated cellular phenomena as well as animal models have been useful to further investigate the specific roles of the virus in disease development.

**Mechanisms of cytomegalovirus-induced transplant vasculopathy**

Transplant vascular sclerosis/vasculopathy, consisting of a concentric intravascular lesion of smooth muscle cells in the vascular intima, is a hallmark of chronic rejection. Smooth muscle cells migrate to the intima and proliferate at this site in the vessel wall. The CMV chemokine receptor molecule US28 provides a molecular link between CMV infection and smooth muscle cell migration, as expression of this molecule induces a massive migration of smooth muscle cells in vitro. When the homologous molecule in rat CMV (R33) was deleted, intimal lesions in a rat transplant model were reduced by ~50%. Thus, a single viral protein appears to be responsible for a substantially enhanced vascular lesion. The US28 also induces production of vascular endothelial growth factor, which could enhance the growth of smooth muscle cells. In infected smooth muscle cells, the
CMV gene IE86 protein binds to p53 and interferes with cell cycle control; this gene may be involved in rapid restenosis after coronary angioplasty\textsuperscript{37} and perhaps in intimal hyperplasia in transplanted grafts.

Another hallmark of vascular disease is the formation of foam cells, and lipids also accumulate in the vascular tree in chronic rejection. The CMV increases the expression of CD36 (a scavenger receptor for oxidized LDL) in infected cells, which correlates with increased cellular uptake of lipids\textsuperscript{38}. Since CMV can alter lipid metabolism and accumulate oxidized LDL in infected cells, it may contribute to foam-cell development and increased lipid retention in both atherosclerosis and transplant vasculopathy.

Can cytomegalovirus affect the development of fibrosis?

Fibrosis is a general feature of inflammation, but no clear specific mechanisms can explain why fibrosis develops as a consequence of inflammation. In a rat transplant model, CMV infection increased the expression of both type I and type III collagens and the accumulation of myofibroblasts, which correlated with enhanced interstitial fibrosis in chronic renal allograft rejection\textsuperscript{39}. Human CMV upregulates matrix metalloproteinase-2 (MMP-2) protein levels and activity in smooth muscle cells but not in fibroblasts\textsuperscript{40}. We found that CMV infection of macrophages especially shuts off MMP-9 expression (unpublished results), but upregulates tissue inhibitor of metalloproteinase 1. These observations suggest that perhaps CMV infection directly affects the composition of the extracellular matrix by influencing the synthesis and degradation of extracellular matrix components. This process may be affected differently, depending on which cell types are locally infected, and may lead to instability of an atherosclerotic plaque or to increased fibrosis. Consistent with this possibility, we recently detected CMV-infected cells in areas of fibrosis in transplanted heart explants with severe fibrosis (Fig. 4).

Can cytomegalovirus precipitate ischemia?

Approximately 70\% of patients with a myocardial infarction have clinical symptoms due to rupture of an instable atherosclerotic plaque and formation of an occlusive thrombus that leads to acute ischemia. The CMV-infected
transplant recipients are at increased risk not only for vasculopathy in the transplanted graft but also for cardiovascular disease\textsuperscript{41,42}. Through mechanisms described above, increased CMV activity in the transplant recipient may contribute to atherosclerosis. In addition, CMV-infected endothelial cells appear to be extremely thrombogenic \textit{in vitro}; when transferred to CMV-infected cultures, platelets immediately become activated and aggregate on infected cells, but not on uninfected cells in the same cultures\textsuperscript{43}. This mechanism involves an evacuation of von Willebrand factor from the infected endothelial cells and is mediated by a late but undefined protein. Foscarnet treatment of CMV-infected cells prevented the expression of this protein and prevented the virus from inducing platelet activation and aggregation. In patients with acute myocardial infarction, CMV RNA was found in blood cells in 15\% of patients but in only 2\% of controls\textsuperscript{44}. Thus, an ongoing infection, possibly in the endothelium, might transfer the virus to blood cells in a proportion of these patients. In theory, the virus may infect endothelial cells in the inflamed/active plaque and thereby help precipitate the cardiovascular event. Such a scenario might be prevented by antiviral treatment if high-risk individuals could be identified.

Cytomegalovirus infection and posttransplant diabetes

Cytomegalovirus appears to increase the risk of posttransplant diabetes mellitus\textsuperscript{41,45}, but it is not known how the virus affects pancreatic function. In congenital infection, CMV can infect \(\beta\)-cells in the pancreas. Since these cells cannot regenerate, their destruction by the virus might lead to diabetes. Furthermore, CMV might induce autoimmune T-cell responses through molecular mimicry between CMV UL57 and GAD65\textsuperscript{46-48}. Autoimmune phenomena are well known in CMV-infected individuals, and a variety of autoantibodies are produced during acute infection episodes. The virus may induce B-cell differentiation through an interaction with toll-like receptor 7/9 on plasmacytoid dendritic cells, which results in high production of IFN\(\alpha\), plasma cell differentiation, and antibody production in the presence of IL-2\textsuperscript{49}. Also, CMV can induce the production of specific autoantibodies against CD13\textsuperscript{50,51}, a structural component of CMV\textsuperscript{52} that is a receptor for CMV\textsuperscript{53}. Virus-associated CD13 appears to be immunogenic in stem cell transplant recipients with CMV viremia or disease\textsuperscript{51}. Moreover, these autoantibodies appear in conjunction with CMV, and high levels of CD13-specific autoantibodies were found in patients with extensive chronic graft-versus host disease, which shares many similarities with autoimmune diseases\textsuperscript{54}. These autoantibodies were not found in CMV-negative patients.

The CD13-specific autoantibodies may be produced as a result of T-cell-mediated activation of clones specific for CMV-derived peptides on antigen-presenting cells. This activation of CD13-reactive, non-tolerant B-cells that have internalized CMV particles containing this antigen leads to the processing and presentation of viral peptides together with HLA class II molecules. This in turn stimulates helper T-cells and results in the production of
CD13-specific antibodies. Continued formation of antibodies against CD13 could give rise to tissue lesions. This hypothesis describes a novel mechanism for the development of autoimmune manifestations in man55.

**Cytomegalovirus and opportunistic infections**

Clinical evidence suggests that CMV infection increases the risk of concomitant infections in transplant recipients. In meta-analyses, prophylaxis against CMV reduced herpes simplex virus and varicella zoster virus infections by 73%, but also reduced bacterial infections by 35% and protozoal infections by 69%56. Several lines of evidence suggest that CMV is immunosuppressive, which is probably why CMV-infected patients are at higher risk for other infections. The virus controls specific immune functions through the action of viral proteins. For example, the CMV proteins US2, US3, US6, and US11 can in different ways inhibit the presentation of HLA class I molecules on infected cells; this would lead to an inability to present microbial peptides to cytotoxic T-cells (reviewed57). At least three viral mechanisms control the expression of HLA class II molecules, which would severely impair activation of cytotoxic T-cells, B-cells and natural killer cells by a lack of T helper cell produced cytokines (reviewed57). Natural killer cells, an important part of the innate immune response, are the first line of defense against viral infections and are also targeted by CMV. Several CMV proteins inhibit the activation of natural killer cells57, and UL16 mediates protection against cytolytic proteins released from cytotoxic T-cells and natural killer cells58. As a result, infected cells are protected against killing58.

Antigen-presenting cells such as monocytes/macrophages and dendritic cells are also directly influenced by the virus. The CMV inhibits the differentiation of monocytes into both macrophages and myeloid dendritic cells and impairs their ability to take up and present peptides to T-cells59-61 and to migrate in response to inflammatory chemokines62,63. Maturation of immature into mature dendritic cells represents a central phenomenon in dendritic cell biology, and is required before these cells can to migrate to lymph nodes. The CMV affects the ability of dendritic cells to mature64, and when mature myeloid dendritic cells become infected, they rapidly release RANTES, macrophage inflammatory proteins 1α and 1β, which bind to their receptors and internalize the receptor complex; as a result, the cells lack CCR1 and CCR5 on the cell surface and their ability to migrate is severely impaired65. In mice, murine CMV infection causes a functional paralysis of dendritic cells66. In heart transplant patients, CMV infection leads to a reduction or a complete loss of dendritic cells in peripheral blood and a severely impaired T-cell response67. Similar findings were reported in immunocompetent individuals with CMV mononucleosis, suggesting that the virus has immunomodulatory effects in the absence of immunosuppressive drugs68.

In contrast, mice latently infected with murine CMV were recently reported to be resistant to infection with the bacterial pathogens *Listeria monocytogenes* and *Yersinia pestis* through a long-lasting activation of macrophages69. This may be an evolutionary benefit of a latent CMV infection in the host. An acute infection would instead impair the immune system, rendering the host unable to eliminate the virus at the cost of increased susceptibility to other infections. However, an induced activation of granulocytes has also been reported in cells infected in vitro, but it is not known whether these cells exhibit an impaired or enhanced ability to kill bacteria70. In addition, leukotriene B4 interacts with the leukotriene B4 receptor 1 on neutrophils and releases antimicrobial peptides in the mouse model71. Although this scenario should im-
prove the immune response to bacterial infections, clinical observations demonstrate increased bacterial infections in CMV-infected transplant patients. Thus, undefined specific mechanisms may explain why CMV-infected patients are more susceptible to bacterial infections.

Cytomegalovirus infection and malignancies

Posttransplant lymphoproliferative disease (PTLD) is the most common malignancy in transplant recipients. It occurs in 1-20% of solid organ transplant patients and has been linked to high immunosuppression and reactivation of Epstein-Barr virus. However, the incidence of PTLD is also increased 7- to 10-fold in patients with CMV disease, and PTLD is associated with CMV reactivation (reviewed). Prophylaxis with ganciclovir, which targets both viruses, reduces the risk of PTLD in organ transplant recipients.

Cytomegalovirus has recently been detected in several malignant tumors, including glioblastoma multiforme, colon cancer, prostatic carcinoma, and breast cancer (personal communication, Dr. Charles Cobbs, California Pacific Medical Center, San Francisco) using the HSIS technique. Although recent evidence suggests that the CMV-encoded chemokine receptor molecule US28 can induce tumors in severe combined immunodeficiency disease mice, CMV is not generally considered to be oncogenic. Instead, it may be an oncomodulator that contributes to oncogenesis by modifying tumor cell biology. The CMV gene products can control different cellular pathways that might be involved in oncogenesis, including cellular differentiation, cell cycle regulation, DNA repair mechanisms, epigenetic functions, apoptosis, cellular migration, angiogenesis, and immune evasion mechanisms.

Thus, a number of CMV proteins protect infected tumor cells from elimination by the immune system and control pivotal cell functions that may be biologically important in the clinical course of several cancers.

In support of this hypothesis, we found an active CMV infection in 61 (98%) of 62 glioblastoma multiforme. Remarkable, patients whose tumors had a low-grade CMV infection or no infection at all lived more than twice as long as patients whose tumors had high-grade infection (unpublished observations). This finding strongly implies a pathogenetic role of CMV in the progression of glioblastoma multiforme, the first disease in immunocompetent individuals that may be linked to this virus. Glioblastoma multiforme is not overrepresented in organ transplant recipients, but the incidence of colon, prostate and breast cancer is higher among these patients. Additional studies will be needed to define the potential role and clinical relevance of CMV in different cancers.

Conclusions

In summary, emerging evidence suggests that CMV not only causes acute clinical disease in transplant recipients, but may also contribute to the pathogenesis of a variety of clinical syndromes after transplantation. In meta-analyses, concomitant infections, acute and chronic rejection, and overall mortality were increased in CMV-infected patients, but were reduced in those receiving antiviral prophylaxis. As exemplified above, many specific CMV proteins and strategies likely contribute to a variety of phenomena that negatively influence the host. As the virus interacts with its host in complex ways, and large numbers of immunosuppressed individuals have only been around for 30-40 years, the virus faces new evolutionary challenges, which may alter the virus in the future. Increased awareness of the capability of this interesting and important human pathogen
Trends in Transplantation 2008;2

will hopefully lead to better control of CMV infections in transplant patients, resulting in better long-term graft and patient survival.

Acknowledgements

We thank Stephen Ordway for editing the manuscript.

Our work is supported by the Swedish Medical Research Council, Swedish Children Foundation, Cancer Foundation and Heart and Lung Foundation.

References