Discussion

Cytomegalic endothelial cells in the pathophysiology of CMV infections

The presence of cytomegalic endothelial cells (CEC) in peripheral blood during human cytomegalovirus (CMV) infections demonstrated vascular involvement during CMV infection [1,2]. These cytomegalic endothelial cells were permissively infected with CMV and in late stage of infection. Owing to their large size, these CEC may plug in capillary vessels and thus play a role in CMV-mediated organ dysfunction. In this thesis we investigate the presence of CEC in the context of vascular damage during CMV infection in kidney transplant recipients and examine the contribution of CEC to the pathophysiology of CMV-induced organ disturbances. Although infection with CMV directly damages endothelial cells, the immunological response induces a massive inflammatory activation as well. Therefore, we propose that the pathophysiology of CMV-induced organ disturbances includes tissue damage caused by CMV infection as well as the inflammatory response to CMV, which is in fact a normal physiological response.

In the second part of the thesis, we describe some aspects of CMV infected endothelial cells in viral dissemination and pathophysiology.

In order to isolate and quantify CEC from peripheral blood of transplant recipients, we developed a method that could serve a dual purpose: that is to quantify and to identify the isolated cells. A FACS method was used by which the mononuclear cell (MNC) fraction including endothelial cells was sorted after they were stained with an endothelial cell (EC)-specific marker. Cells were sorted onto adhesion slides, after which the sorted cells could be identified by staining with CMV specific markers. The recovery percentages and reliability of the FACS method are described in Chapter 2 and compared to the conventional method relying on the cytocentrifugation of the isolated MNC fraction. In short, the FACS method was comparable in recovery but more sensitive. For reasons of logistics, in the longitudinal patient study we preferred the cytocentrifugation method to the FACS sorting method.

In a cohort of 54 kidney transplant recipients followed during the course of CMV antigenemia we detected CEC only in the patients with moderate or high CMV antigenemia levels. The relationship between the height of the viral load and the observed frequencies of CEC in our study differed from the literature. This depended on factors such as the type of transplantation (i.e. kidney, bone marrow, heart), the use of preemptive HCMV therapy and the applied immunosuppressive regimen [2,3], which may influence the replication rate of CMV in endothelial cells. We infer that these circumstances may induce a preference of CMV to replicate in endothelial cells or that they affect the detachment process of endothelial cells.

Uninfected endothelial cells in blood

In addition to CEC we detected endothelial cells (EC) that were negative for all CMV replication-stage specific markers that were tested. Strikingly, as for CEC, the uninfected EC appeared during or shortly after the maximum levels of CMV antigenemia. The direct CMV infection of endothelial cells results in late stage infected EC in blood. However, the release
of the uninfected endothelial cells in blood must be influenced by other parameters as well, since EC were also observed in patients without CMV infection. Endothelial cells circulating in blood have been described for several abnormalities with vascular injury, such as during infection with *Rickettsia coronii* [4], sickle cell anemia [5] or in coronary diseases [6]. They were activated [7] and most EC were not apoptotic [5,6], although doubted by others [8,9]. It was supposed that the EC originate from the lesion sites in the endothelial layer. However, the exact mechanism leading to the detachment of these cells remains largely unknown.

Recently, a “new type” of circulating endothelial cells in blood has been described [10], i.e. CD34 positive progenitor cells originating from the bone marrow. In the presence of appropriate growth factors, the CD34 positive progenitor cells can differentiate into endothelial cells [10]. In the recovery phase of vascular lesions, the release of growth factors into blood, such as VEGF, stimulates CD34 positive progenitor cells to home to a vascular lesion and to differentiate into an endothelial cell [11]. In a study with bone marrow transplant patients, two genotypes were identified of EC from blood, the genotype from the recipient, thus vessel-derived, and the genotype of the donor that is bone marrow derived. Especially, the bone marrow derived EC had a large proliferation capacity in vitro [12].

The uninfected EC observed during CMV infection were stained for several markers (Chapter 3). Unlike the CD34 progenitor cells, which were described to be CD45 positive, the EC isolated during CMV infection, were CD45 negative. Therefore, we assumed that they were not bone marrow derived. It would appear likely that the EC were released from the vasculature due to acute rejection or a CMV induced process. With our method of isolation and staining with monoclonal antibody E1/1 2.3 we detected no uninfected endothelial cells in healthy individuals or in patients at moments other than during CMV infection or acute rejection. Therefore we doubt whether EC progenitors from the bone marrow can be detected with our monoclonal antibody. Nevertheless, identification and purification of endothelial progenitor cells in transplant recipients may be of particular importance in the context of the recovery mechanisms of damaged endothelial lesions, as is the case after detachment of CMV infected endothelial cells.

**Relation with acute rejection**

In our study, almost all kidney transplant recipients with either CEC, EC or both in blood during active CMV infection had suffered from acute rejection. The relationship between the occurrence of (C)EC during CMV infection and preceding acute rejection was also reflected by a substantial increase in the levels of markers for endothelial damage Von Willebrand Factor (VWF) and soluble vascular cellular adhesion molecule-1 (sVCAM-1). Although CMV infection and acute rejection are both associated with vascular damage (Chapter 3 and 4, [13,14]), enhancement during CMV infection due to preceding acute rejection episodes was not reported before. In a rat model, allogeneic lung transplantation followed by CMV infection showed that cytomegalovirus infection and acute rejection reinforced each other. This process was reflected by higher expression levels of the adhesion molecules ICAM-1 and VCAM-1 as well as their ligands LFA-1 and VLA-4 and MHC class II in the graft[15].
However, the rejection and CMV infection occurred synchronous, which differed from the situation in our patients. In the patients we observed a lag time between rejection and infection.

Another intriguing issue is the localization of endothelial injury. Acute rejection is directed at endothelial cells in the transplanted graft. In contrast, CMV may cause a systemic viral infection that involves many organs. The CEC that are detected in blood obtained from the forearm, are thought to originate from (capillary) vessels in the hand or lower part of the arm [1]. Thus, acute rejection episodes in the graft sensitizes the endothelial surface of the host to HCMV induced vascular injury.

An argument to explain the enhancement of endothelial damage during CMV infection after acute rejection as well as the probable difference in location of endothelial damage between acute rejection and CMV infection could be that infection of the endothelium with CMV merely enhances the already ongoing damage caused by acute rejection. However, it made no difference whether the time span between the onset of rejection and diagnosis of CMV infection was short or long i.e. a long time span was not associated with a lower increase of VWF during CMV infection. Moreover, basal levels of VWF and sVCAM-1 of patients with acute rejection before CMV infection were not higher than those of CMV patients without an acute rejection. Yet, the baselevels were substantial higher than those of healthy individuals. Another explanation could be that acute rejection before CMV infection may cause a more serious infection and thus more endothelial damage. The extent of vascular damage was indeed related to the severity of infection. However, within the group of patients with severe CMV infection, the patients with preceding rejection episodes had significantly more CEC and EC as well as larger increases of VWF and sVCAM-1. Evidently, a preceding acute rejection increases the susceptibility of endothelial cells to CMV-induced damage.

The enhanced vascular damage as well as the presumably different locations of vascular damage during CMV infection or acute rejection could be caused by the induction of antibodies against epitopes of (activated) endothelial cells. These anti-endothelial cell antibodies should then have been developed during or after acute rejection. Anti-endothelial cell antibodies (AECA) have been described to circulate in blood of kidney transplant recipients and can be produced after CMV infection [16]. Furthermore, they are suggested to have a role in chronic transplant dysfunction [17]. However, these AECA have to be induced in the short interval between acute rejection and CMV infection. Furthermore, they need to be of low affinity; otherwise a complement-mediated hyperacute rejection will be induced, which is in contrast to the subtle amounts of EC in blood that we observed during CMV infection. The presence and function of these AECA can be investigated by incubation of activated in vitro cultured endothelial cells with patient sera drawn after acute rejection. Binding of IgM or IgG can be detected by FACS staining or in a complement assay.

Alternatively, the enhanced vascular damage during CMV infection might be influenced through the intracellular transcription pathway. One of the first events in an inflammatory response, such as in acute rejection, is release of proinflammatory cytokines. These cytokines activate endothelial cells to produce chemokines and cytokines. Leukocytes are attracted and as a consequence they will engage contact. In particular, the binding of TNFα to its receptor results in activation of a number of transcription factors. Among these,
activated NFκB plays an important role in the regulation of genes for endothelial activation. In addition, NFκB can bind to and activate the CMV IE promoter/enhancer region as well [18,19]. Thus the activation of NFκB enhances the infectivity of CMV in the infected cell. Reactive oxygen species [19], released by CTLs or macrophages or oxidized low-density lipoproteins [20] activate the signaling cascade leading to the NFκB-mediated enhancement of CMV replication as well. Obviously, as is shown by in vitro studies, CMV replication has benefit of a pro-inflammatory environment. In transplantation patients, high plasma levels of TNFα were correlated to high CMV antigenemia values [21]. This might be due to the fact that the susceptibility of a cell to CMV infection after acute rejection is caused by a (structurally) increased activation (or susceptibility to activation) in the nucleus. The elevated basal levels of VWF, sVCAM-1 and sICAM-1 support the observation of a higher activation level after transplantation together with observations of the increased expression of adhesion molecules at tissue level. Kidney biopsies obtained before and after acute rejection revealed an increased expression of adhesion molecules as well [22], thus in the graft a higher status of activation is induced.

**Implication for chronic transplant dysfunction**
The acute rejection-mediated enhancement of vascular damage during CMV infection raises the question whether other events that are harmful to the endothelial surface can contribute to a build-up of vascular damage as well, which may end in chronic transplant dysfunction. Throughout the transplantation procedure and follow-up, the recipient meets many insults with a high risk in view of the graft and vasculature. It has already started in the history of kidney failure and dialysis followed by the transplantation with surgery, ischemia and subsequent reperfusion of the transplanted kidney [23]. In terms of medication, cyclosporin A has nephrotoxic side-effects [24,25], whereas prednisolone is associated with a disturbed lipid profile [26]. A number of transplant recipients suffer from acute rejection, bacterial or viral infections [27]. Cold ischemia, acute rejection, CMV infection and hyperlipidemia are all risk factors for the development of chronic transplant dysfunction [28,29]. Presently, no effective treatment exists for chronic transplant dysfunction. As discussed before, if multiple factors after transplantation indeed accumulate their harmful effect at the endothelial surface, too many hits after the transplantation may be fatal for the graft some years after the transplantation.

**CEC in relation to CMV induced pathophysiology**
To study the role of endothelium, in particular the CEC, in the pathophysiology of CMV-induced organ disturbances, we investigated whether the cellular (endothelial cells in blood) and humoral (VWF and sVCAM-1 levels) parameters were related to the clinical symptoms in the patients with CMV infection. CEC, EC or both were more frequently detected in patients with clinical symptoms. There was no significant relationship between increases in levels of VWF, sVCAM-1 or sICAM-1 and CMV-induced clinical symptoms.
In patients the developing clinical symptoms were frequently observed during increasing CMV antigenemia levels. The CEC typically appeared at or around the maximum values or 3 – 10 days afterwards. Thus, the kinetics of appearance of CEC in blood indicated that they could not be responsible for the CMV induced symptoms.

The mean number of CEC in the patients with CEC was 2.6 CEC per ml, which is 13,000 CEC in 5 liter blood. Probably the concentration is too low to cause detectable disturbances by plugging of CEC. One reason could be that the blood vessels have dilated their lumen to compensated obstruction induced by CEC, because they have a large potential in regulating the vascular tone. One of these comprises adenosine, a potent vasodilator [30]. CMV infected endothelial cells in vitro express higher levels of ecto-ATPase and ecto-5‘ nucleotidase, which locally may cause increased levels of adenosine.

As an alternative, the CEC may become ruptured in the small capillaries. In vivo, the CMV-induced organ dysfunction was investigated by measurement of the pulmonary diffusion capacity of the lungs. Van Son et al. reported that kidney transplant recipients had a significant decrease of the pulmonary diffusion capacity during CMV infection, even if they had no evident clinical symptoms [31]. Furthermore, disturbances in organ function induced by CMV could also be found for the intestines [32]. The next step was to identify the role of CEC in these CMV-induced organ function disturbances. For this, a series of 26 kidney transplant recipients of which 13 patients with CMV infection underwent serial pulmonary diffusion tests in the course of their CMV infection. The contribution of for instance inflammation and swelling, fibrosis, fluid overload as well as the blood flow could be determined by measurement of the specific carbon monoxide diffusion capacity. The CO diffusion capacity has two components: the pulmonary diffusion membrane factor (Dm) and the pulmonary capillary blood volume (Vcap). We found that not only the Vcap was reduced but also the Dm. Therefore, a reduction in blood volume caused by obstruction of the capillary vessels due to plugging of CEC could not be the only explanation for the decrease in pulmonary diffusion (Chapter 5.1).

Nine of fifteen CMV patients that underwent pulmonary diffusion tests were also studied for the occurrence of CEC. However, between patients with or without CEC no differences were observed in either Vcap or Dm. Moreover, higher levels of CMV antigenemia were positively related to a decrease of Dm. Thus, it is unlikely that plugging of CEC is responsible for the decrease in pulmonary diffusion. Obviously, the level of pneumonitis is related to severity of infection by means of a local inflammatory process that caused a lower Dm. However, if CEC become ruptured in the capillaries, they may release a large amount of infectious virus. Released viral particles may induce new sites of infection and thus enhance local inflammation. Since CEC occur at moderate to high CMV antigenemia levels, the association of CEC with decreased pulmonary diffusion is probably indirect and mediated by the severity of the infection. Thus, both approaches to identify the contribution of CEC to CMV-induced organ pathophysiology indicated that a direct role of CEC by plugging is not likely.
CMV infected endothelial and viral pathophysiology

In the second half of this thesis we studied CMV infected endothelial cells as a source for viral proteins and infectious virus in polymorphonuclear cells (PMN) (Chapter 6+7) as well as some aspects of endothelial pathophysiology during CMV infection (Chapter 8). Apart of the late-stage infected CEC in blood, the CMV pp65 positive PMN is a likely candidate to disseminate the virus in vivo. The use of endothelial adapted CMV strains or low-passage clinical strains of CMV to infect endothelial cells, enabled the generation of pp65 positive PMN after coculture with CMV infected endothelial cells or fibroblasts [33,34].

We used this technique to study the development of CMV antigenemia in vitro. We examined the mechanism of uptake of viral proteins and particles, strategies to interfere in the transfer of pp65 from infected endothelial cells to PMN and the possibility that the PMN deliver infectious virus to uninfected monolayers.

Since phagocytosis is a main function of PMN, it seemed the most plausible mechanism to obtain viral proteins and particles released from CMV infected endothelial cells. Furthermore, by electron microscopy analysis of monolayers of infected endothelial cells in the presence of PMN, we observed PMN near endothelial cells that engulfed CMV dense bodies. However, as described in Chapter 7, uptake of pp65 by phagocytosis only was less likely. Some of the pp65 positive PMN had formed clusters of fused cells, with separated nuclei or nuclei containing a large clump of nuclear lobes. Beside phagocytosis for uptake of pp65, two arguments favor fusion of the PMN with viral particles in the presence of or induced by contact with CMV infected endothelial cells as a mechanism. In the first place, the viral particles fuse with PMN but also trigger the PMN to fuse with the neighboring PMN leading to formation of syncytia, and second, CMV specific neutralizing antibodies from patient sera inhibit the uptake of pp65.

Especially the role of CMV specific antibodies is of great interest for the development of CMV antigenemia in vivo. They could mask the local viral load, i.e. CMV infected endothelial cells in the body, at sites were PMN would normally acquire pp65. Moreover, CMV specific antibodies reduce the generation of pp65 positive PMNs. In vitro it has been demonstrated that pp65 positive PMNs can retransmit the virus to uninfected cells [34]. It is likely that CMV specific antibodies inhibit the CMV antigenemia-mediated viral dissemination in vivo too.

An element of endothelial activation involves the transition from an anti-thrombotic surface to a more prothrombotic state, which facilitates the adherence of platelets and conversion of fibrinogen to fibrin. The development of an irregular surface of the endothelial cells reduces the blood flow, providin a better access of leukocytes to activated endothelial cells and underlying tissues. For this, ecto-ATPases (CD39) are downregulated, because the conversion of ATP and ADP to AMP prevents recruitment and activation of more platelets. Adenosine formed from AMP by ecto-5’nucleotidase (CD73) in turn has anticoagulant and anti-inflammatory properties. Thus the balance between ecto-ATPase and ecto-5’ nucleotidase is critical for maintenance of the anti-thrombotic state.

We found that the expression of ecto-ATPase and ecto-5’ nucleotidase was upregulated on CMV infected endothelial cells, in contrast to the pattern expected during activation of endothelial cells. Obviously, CMV has, in parallel to other evasion strategies, modified the
procoagulant features of the inflammatory response to circumvent a vigorous immunological response of the host leading to rapid clearance of the virus.

**Conclusion and directions for further investigation**
The role of CMV infected cells is important in the acute phase of CMV infection, but may also contribute to development of chronic transplant dysfunction. Different from our hypothesis, the contribution of CEC in blood to CMV-induced clinical symptoms by plugging is not detectable. However, the occurrence of CEC and EC in blood was associated with CMV clinical symptoms. It is likely that this relationship is mediated indirectly and is linked via the shared relationship with the severity of CMV infection, i.e. the CMV antigenemia levels or viral load.

*In vitro* experiments indicate that CMV infected endothelial cells probably have a more important role in the infection, before they detach and are released into the circulation. CMV infected endothelial cells may deliver viral particles and proteins to PMN, resulting in CMV pp65 positive antigenemia. Once the PMN have taken up the virus, they can transmit infectious virus to uninfected endothelial cells or other target cells at different sites in the body. Whether this may happen *in vivo* is a topic for investigation, which is currently tested in a rat model with RCMV (BWA van der Strate personal communication).

It could be possible that apart of the combination acute rejection – CMV infection, other combinations induce enhanced vascular damage as well. For instance, the duration of cold ischemia or the kidney function as reflected in creatinine levels can be related to the severity of endothelial damage during acute rejection and CMV infection. Cellular parameters (CEC and EC counts in blood) and humoral parameters (VWF, sVCAM-1 or others) may reveal information about the extent of vascular activation and damage during CMV infection. Of these, measurement of the humoral parameters is probably sufficient to study these questions, whereas the analysis of CEC and EC counts is rather laborious and is therefore not recommended for a large scale study of vascular damage. Although VWF and sVCAM-1 are released by different mechanisms, determination of the plasma levels like VWF, sVCAM-1, PAI-1 and tPA (coagulation and activation parameters, not endothelial specific) [35] or thrombomodulin (endothelial specific, sensitive to endothelial activation and damage) [35] may give evidence about enhanced endothelial damage after for instance severe reperfusion injury and CMV infection.

At the cellular level, it would very interesting to determine the factors involved in the enhanced susceptibility, such as levels of activated NFκB after repetitive injury or insults to *in vitro* cultured endothelial cells. Furthermore, the expression of adhesion molecules and production of growth factors may indicate the level of activation and injury. A drawback is that *in vitro* cultured endothelial cells continuously grow and thus have angiogenic features, whereas *in vivo* the endothelial cells are in a resting state. Kidney biopsies obtained during or after CMV infection can be examination for the expression levels of growth factor receptor, molecules involved in the remodeling of vessels after vascular lesions, such as matrix metalloproteases, or collagen production. This may give important information about the
contribution of CMV induced vascular damage to the development of chronic transplant dysfunction in the long run.

A small-scale study of circulating endothelial cells in blood could give information whether the uninfected endothelial cells are donor or recipient derived. Donor derived endothelial cells may indicate that probably rejection is involved in the release of these cells. In addition, the question can be answered whether the circulating uninfected endothelial cells are apoptotic or not. If so, they are probably released from the damaged lesion and they will not be derived from the bone marrow as endothelial progenitor cells.

It would be interesting to study the recruitment of CD34 positive endothelial progenitor cells during CMV infection, acute rejection or after reperfusion of the graft. Using the FACS sorting method (Chapter 2) blood samples could be screened and the isolated cells could be stained with for instance endothelial differentiation markers. Especially after reperfusion or acute rejection, the bone marrow derived pro-angioblasts may target to and replace endothelial cells from damaged lesions inside the graft, thus reducing the antigenic area.

References


