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Mechanisms of Cytomegalovirus-Accelerated Vascular Disease: Induction of Paracrine Factors That Promote Angiogenesis and Wound Healing

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Abstract

Human cytomegalovirus (HCMV) is associated with the acceleration of a number of vascular diseases such as atherosclerosis, restenosis, and transplant vascular sclerosis (TVS). All of these diseases are the result of either mechanical or immune-mediated injury followed by inflammation and subsequent smooth muscle cell (SMC) migration from the vessel media to the intima and proliferation that culminates in vessel narrowing. A number of epidemiological and animal studies have demonstrated that CMV significantly accelerates TVS and chronic rejection (CR) in solid organ allografts. In addition, treatment of human recipients and animals alike with the antiviral drug ganciclovir results in prolonged survival of the allograft indicating that CMV replication is a requirement for acceleration of disease. However, although virus persists in the allograft throughout the course of disease, the number of directly infected cells does not account for the global effects that the virus has on the acceleration of TVS and CR. Recent investigations of up- and down-regulated cellular genes in infected allografts in comparison to native heart has demonstrated that Rat-CMV (RCMV) up-regulates genes involved in wound healing (WH) and angiogenesis (AG). Consistent with this result, we have found that supernatants from HCMV infected cells (HCMV secretome) induce WH and AG using *in vitro* models. Taken together these findings suggest that one mechanism for HCMV acceleration of TVS is mediated through induction of secreted cytokines and growth factors from virus-infected cells that promote WH and AG in the allograft, resulting in the acceleration of TVS. We review here the ability of CMV infection to alter the local environment by producing cellular factors that act in a paracrine fashion to enhance WH and AG processes associated with the development of vascular disease, which accelerates chronic allograft rejection.

1 Introduction

The importance and interest of HCMV as a pathogen has increased over the last few decades, with the escalation in the number of immunosuppressed patients, either undergoing immunosuppressive therapy following solid organ or bone marrow transplantation, or with

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AIDS patients. Primary HCMV infection is followed by a life long persistence of the virus in a latent state and reactivation of latent virus is considered to be the major source of virus in immunocompromised individuals (Reeves and Sinclair, this volume). HCMV is linked to the development of arterial restenosis following angioplasty, atherosclerosis, and solid organ TVS (Melnick et al. 1983; Speir et al. 1994; Melnick et al. 1998). HCMV infection nearly doubles the 5-year rate of cardiac graft failure due to accelerated TVS (Grattan et al. 1989) and prior to the advent of ganciclovir therapy, doubled the rate of liver graft loss at 3 years (Deotero et al. 1998; Rubin 1999). In recipients of heart transplants, treatment with ganciclovir, a potent inhibitor of viral replication and CMV disease, delayed the time to allograft rejection (Merigan et al. 1992). A subsequent post-hoc analysis of these data confirmed that prophylactic ganciclovir treatment delayed graft rejection compared to controls (Valantine et al. 1999). Moreover, the early control of sub-clinical HCMV replication after cardiac transplantation by T-cell immunity reduces allograft TVS and CR (Tu et al. 2006). Additional proof to HCMV's affect on graft TVS comes from the finding of a higher incidence of viral DNA detected in the explant vascular intima of those patients with cardiac allograft TVS than in those explants without vasculopathy (Wu et al. 1992). In fact, the mere presence of HCMV infection in kidney transplant patients, whether displaying asymptomatic or overt symptoms, was shown to negatively impact allograft survival (Fitzgerald et al. 2004). While a number of studies have provided strong evidence for a role of HCMV in the development of TVS and accelerated CR, the precise mechanisms involved in this process are still unknown.

2 Tissue repair and angiogenic factors mediate TVS

Despite recent medical advances, the long-term survival of solid organ allografts has not improved, largely due to CR. The high prevalence of CR is of particular concern given that to date the only effective therapy is re-transplantation. The primary component of CR is an accelerated form of arteriosclerosis known as transplant vascular sclerosis (TVS). TVS is characterized histologically by diffuse concentric intimal proliferation that ultimately occludes the vessel (Billingham 1992). A growing body of evidence supports a role for angiogenesis and tissue repair processes in the development of vascular disease (Shibata et al. 2001; Khurana and Simons 2003). A sparse number of macrophages, T-cells, NK cells, and B-cells are seen in early lesions, while late lesions are associated with a thickened intima containing SMC interspersed with macrophages (Clinton and Libby 1992). Activated inflammatory cells and SMC within and near vascular lesions are important local sources of pro-angiogenic factors (Bouis et al. 2006). TVS development involves chronic perivascular inflammation, EC dysfunction, and SMC migration from the media to the intima and proliferation that result in deposition of ECM and neointimal thickening of the allograft arterial wall (Libby et al. 1989; Billingham 1992; Hosenpud et al. 1992). These events result in vessel narrowing, occlusion, and graft failure. The pathological processes involved in TVS and other vascular diseases are akin to many of the cellular events that mediate normal tissue repair/angiogenesis. In all cases, a complex interaction between cells and surrounding regulatory factors, enzymes, and ECM components leads to cellular migration, proliferation, and tissue remodeling.

The formation of new blood vessels or angiogenesis is broadly divided into the following three phases: vessel destabilization, proliferation/migration and vessel maturation (Carmeliet 2003). In normal adults, angiogenesis is normally restricted to formation of placental and endometrial tissue, hair follicle vascularization and tissue repair. During these processes the endothelium remains inactive due to a balance of positive and negative regulatory factors. When vessel growth is required, the regulatory balance tips toward pro-angiogenic factors. Restoration of steady state is achieved by increasing angiogenesis inhibitors and vessel stabilization factors. Breakdown of the tightly regulated angiogenic balance leads to abnormal angiogenesis and contributes to a variety of pathological disorders, including cancer, autoimmune conditions, and cardiovascular disease (Carmeliet 2003). Leukocytes play a role

in normal angiogenesis through contribution of pro- and anti-angiogenic factors, but are particularly important in pathological (immune- or tumor-driven) angiogenesis (Kent and Sheridan 2003).

Wound healing (WH) is a complex process involving the sequence of inflammation, tissue formation and tissue remodeling which results in the reestablishment of an anatomical or physiological barrier (Kent and Sheridan 2003). Wound healing involves a local milieu created by the coordinated action of growth factors, cytokines, enzymes and extracellular matrix (ECM) components, interacting with the injured tissue. In addition, an influx of inflammatory cells is involved in this process. Angiogenesis itself is an important component of WH. Given the role of inflammation, new vessel growth and tissue remodeling in WH, it is not surprising that many of the same stimulatory and inhibitory factors promote both angiogenesis and WH (Werner and Grose 2003).

3 Animal models of CMV-accelerated graft rejection

Determining the mechanisms involved in the development of HCMV-associated TVS has been difficult because the etiology of this disease is multifactorial. In addition, HCMV is ubiquitous throughout the human population, and hence negative controls are rare. Furthermore, HCMV infections are lifelong during which time the virus infects all of the cell types involved in TVS formation including SMC, EC, macrophages, and fibroblasts. Along these lines, HCMV evades the immune system by remaining latent in monocytes and HCMV reactivation in immunocompetent hosts is difficult to detect when clinically silent (Lemstrom et al. 1993; Bruning et al. 1994; Lemstrom et al. 1995; Orloff 1999; Orloff et al. 2000). These undeniable factors make it difficult to determine a temporal relationship between the virus infection and TVS, and because of obvious ethical reasons, human studies are impossible. Therefore, animal models provide an ideal tool to study the association between CMV and TVS. In fact, the most compelling evidence that herpesvirus infections play a role in the vascular disease process is exemplified through the use of animal models. For example, in rat solid organ transplantation, acute infection with rat (R)CMV infection accelerates TVS, which leads to untimely graft failure (Orloff et al. 2002; Streblow et al. 2003; Soule et al. 2006). Similar to the human transplantation setting antiviral therapy reduces the acceleration of rejection in rat transplant models demonstrating that active CMV replication is required for these disease processes (Tikkanen et al. 2001a; Zeng et al. 2005). Importantly, the effects of RCMV on the acceleration of TVS are not organ-type specific but can occur in a broad range of solid organ transplants including heart, kidney, lung, and small bowel (Tikkanen et al. 2001b; Orloff et al. 2002; Streblow et al. 2003; Soule et al. 2006).

3.1 RCMV accelerates TVS & CR in a rat heart transplantation model

In order to study the role of CMV in the development of TVS, we have taken advantage of the F344 into Lewis rat heterotopic solid organ transplant model (Ely et al. 1983; Klempnauer and Marquarding 1989; Lubaroff et al. 1989), which we have used to study TVS/CR in transplanted heart, kidney and small bowel grafts (Orloff et al. 2002; Streblow et al. 2003; Streblow et al. 2005; Soule et al. 2006; Streblow et al. 2007). Since this strain combination exhibits reduced allogenicity, acute rejection is prevented by a short regimen of cyclosporine A, resulting in long-term surviving allografts developing histological evidence of CR (Orloff et al. 2002). Heart allograft recipients not treated with CsA acutely rejected, and syngeneic transplants failed to develop TVS/CR. In the CsA treated heart allograft recipients, the mean time to develop CR was 90 days, as determined by palpation of the abdomen for induration of the graft and diminished pulsation of the heartbeat. The majority of the vessels in the rejecting cardiac allografts showed the presence of TVS.

Using this rat heart transplant model, we have determined that RCMV accelerated the time to develop TVS and graft failure from 90 to 45 days and increased the degree of TVS from a mean neointimal index (NI)=42.9 to 82.9 (Streblow et al. 2003). RCMV infection failed to induce TVS in syngeneic cardiac grafts by the study endpoint of 120 days (mean NI=4.2 versus uninfected controls mean NI=8.8). Assessment of grafts at earlier times before CR has revealed an even more pronounced effect of RCMV infection on TVS progress. RCMV infection significantly increased the severity of TVS in heart allografts at days 28, 35 and 45 (mean NI=48, 70, & 82) compared to uninfected recipients (mean NI=31, 30 & 43). Graft hearts from infected but not uninfected recipients showed an early (days 7 & 14) presence of endothelialitis (Streblow et al. 2003). In these animals, PCR identified RCMV DNA in all submandibular glands (SMG), at days 7, 14, 21, 28, 35 and 45. In native and graft hearts, RCMV DNA was highest at post-operation days 7 and 14, corresponding to the time when endothelialitis is present within the graft vessels. After this time virus production is low but detectable throughout the development of TVS (Streblow et al. 2003). However, RCMV was only detected in the blood until 14 days. The presence of virus in the heart allografts at the later time points post infection was confirmed by immunostaining for RCMV-IE proteins. The number of RCMV IE positive cells present in the allografts at days 21 and 28 was low, however, positive cells are observed as single, infected cells or in small 10–30 infected cell foci. To determine whether virus replication was necessary for RCMV-accelerated TVS, we treated allograft recipients with ganciclovir (20mg/kg/day for 45 days), which increased the mean time to allograft CR from 45 to 75 days ($p<0.001$) and decreased TVS formation from a mean neointimal index of 80 to 65 ($p<0.001$).

In the rat heart transplant model, using a bone marrow chimera model of tolerance induction, the recipient alloreactive immune response was shown to be required for RCMV-acceleration of graft loss (Orloff et al. 2002). These data suggest that RCMV infection requires an allogeneic immune environment for the acceleration of TVS. In this rat cardiac transplant CR model, chemokine expression was increased in virus-infected recipient grafts compared to uninfected controls (Streblow et al. 2003). RCMV-infected graft tissues also contained increased numbers of immune cell infiltrates including macrophages, CD4 and CD8 T-cells, and the presence of these cells paralleled the up-regulation of chemokines. Similar findings were observed in a rat CR small bowel and renal transplant models (Orloff et al. 2002; Soule et al. 2006). Our results indicate that while not all allograft cells are infected, viral replication is important to drive the acceleration of TVS. Taken together, our findings suggest that the virus-mediated acceleration of TVS occurs through altered regulation of inflammation and wound healing processes.

3.2 RCMV induces allograft AG and WH genes during the acceleration of TVS

To determine the mechanisms by which RCMV accelerates the development of allograft TVS, we analyzed the cellular RNA expression in allograft hearts with and without CMV infection using DNA microarrays. For these experiments, Lewis recipients of F344 allograft hearts were infected with RCMV; and graft and native hearts were harvested at 21 and 28 days post-transplantation (the critical time of RCMV-accelerated TVS). The allograft hearts from 3 recipients (+/−RCMV) were analyzed using the Affymetrix Rat Genome 230 2.0 arrays with probe sets for 30,000 individual transcripts. The gene expression was analyzed and fold changes were calculated by comparing the native hearts to the infected or uninfected allografts. As anticipated, we identified a number of cellular genes expressed in allografts (infected and uninfected) that were significantly up- or down-regulated (>2-fold) compared to native hearts. While comparing the infected vs. uninfected allografts, we found 385 cellular genes significantly deregulated (>2-fold) at day 21 and 143 such genes at day 28. Interestingly, many of the up-regulated genes are involved in WH including genes associated with tumor invasion, cytokines/chemokines, cytoskeleton, signaling, adhesion and ECM. Approximately, 134 of the up-regulated genes are known mediators of AG/WH including angiopoietin, cathepsins,

chemokines, CNN family, endothelin, ECM/BM components (laminin, fibronectin, osteopontin, tenascin); EGF family; hematopoietic growth factors, IGFBP family, MMPs; PDGF; TGF-β; TNFR superfamily; uPA/uPAR, and VEGF. Shown in Table 1 are the average fold changes of the AG/WH genes from the infected or uninfected allografts compared to the average intensities of the native hearts. The most highly induced genes are part of a signaling complex involved in ECM modification during WH. Of these genes, MMP12 and osteopontin were up-regulated at day 28 in the infected allografts compared to uninfected allografts (367- and 395-fold vs. 6- and 12-fold, respectively, compared to native hearts). Another set of key players of WH is urokinase-type plasminogen activator (uPA) and receptor (uPAR), which were also highly up-regulated in infected allografts compared to uninfected (65 and 12-fold vs. 9 and 3-fold, respectively). In addition, MCP-1 and IL-1, which are potent inducers of MMP12, osteopontin and the uPA system, are highly represented in the infected allografts. Overall, our findings support a hypothesis that RCMV infection tips the balance of activator and inhibitory effectors that drive WH, the result of which is acceleration of vascular disease.

4 *In vitro* models of HCMV mediated wound healing and angiogenesis

While *in vivo* animal models have provided solid evidence for the link between CMV and the acceleration of vascular disease processes, *in vitro* models allow one to explore underlying molecular and cellular mechanisms associated with this link. Vascular tissue repair during allograft rejection involves the cellular processes of migration, activation, proliferation and differentiation and these events occur in multiple cell types including macrophages, EC, SMC and fibroblasts. *In vivo* and *in vitro*, HCMV infects all of these cell types, and aside from immunologic clearance, viral infection modifies many of the host cellular functions that promote tissue repair. For example, CMV infection resulting in acceleration of CR is the increase in the host immune response to the allograft and the virus resulting in recruitment of inflammatory cells, and inflammatory effectors such as chemokines and cytokines including IFN-γ, TNF-α, IL-4, IL-18, RANTES, MCP-1, MIP-1α, IL-8, and IP-10 (Almeida et al. 1994; Almeida-Porada et al. 1997; Vieira et al. 1998; Humar et al. 1999; Streblow et al. 1999; Streblow et al. 2003; Vliegen et al. 2004). CMV also encodes CC and CXC chemokine homologues (Beisser et al., this volume) that recruit a multitude of host cellular infiltrates (Sparer et al. 2004; Noda et al. 2006). In addition, CMV modifies a number of other cellular factors involved in angiogenesis and wound repair processes including adhesion molecules (ICAM-1, VCAM-1, VAP-1, and E-selectin,) and growth factors and receptors (TGF-β, PDGF-AA, VEGF, and PDGFR) (Shahgasempour et al. 1997; Burns et al. 1999; Zhou et al. 1999; Inkinen et al. 2003; Helantera et al. 2005; Inkinen et al. 2005; Reinhardt et al. 2005a; Reinhardt et al. 2005b; Helantera et al. 2006). In addition, increased matrix metalloproteinase (MMP)-2 activity is observed in HCMV infected cells in conjunction with a reduction in matrix gene expression, resulting in a malleability to SMC migration, an alteration in vessel remodeling which promotes a vasculopathy (Schaarschmidt et al. 1999; Reinhardt et al. 2006). The up-regulation of agents that initiate endothelial adhesion and those that promote wound healing provides a means for CMV to enhance the adherence and infiltration of inflammatory cells that drive vascular disease.

4.1 What factors constitute the HCMV secretome?

We and others have demonstrated that HCMV infection alters the types and quantities of bioactive proteins released from infected cells, which we designate as the HCMV secretome. Many of these factors have important roles in vascular disease, and we hypothesize that a major role of CMV infection in the acceleration of TVS occurs through the increased production of WH and AG factors in the allograft. However, neither the complete proteome of the HCMV secretome nor its effects on WH and AG are known. Therefore, in order to determine the effects of HCMV infection on the extracellular milieu, we generated secretomes from HCMV-infected

and mock-infected fibroblasts and determined their protein contents (proteomes) by gel-free LC-MS/MS at Pacific Northwest National Laboratory (PNNL, Richland, WA) and by specific protein arrays. In our LC-MS/MS analysis of the HCMV-infected and mock-infected secretomes, we identified >1,200 proteins with 800 having 2 or more peptide hits. Of the proteins identified by MS/MS, >1,000 were specific or highly enriched in the HCMV secretome, >260 proteins were common to both the HCMV- and mock-infected secretomes, and >225 were specific for the mock-infected secretome. We detected 10 viral proteins in the HCMV secretome of which only 4 were identified with >2 peptides including UL32 (pp150), UL44, UL122, and UL123. Overlaying pathway information and literature mining results, it was noted that a cluster of proteins were involved in integrin signaling and that this cluster was enriched for laminins. Laminins are widely distributed ECM proteins involved in cell adhesion signaling and have recently been implicated in playing a fundamental role in angiogenesis by directly affecting gene and protein expression profiles (Folkman 2003). TGF-β signaling and angiogenesis pathways were also identified in this initial screen.

We also assayed for changes in 174 common cytokines/growth factors present in the HCMV secretome using RayBio® Human Cytokine Array G Series 2000 antibody arrays. We analyzed secretomes from HCMV- and mock-infected fibroblasts. We detected 144 of the 174 factors in the HCMV secretome when our cut off value for detection was set at an average intensity of 500. Of these 144 proteins detected, 41 factors were significantly induced over the mock-infected secretome (Table 2). The 35 most predominant proteins detected in the HCMV secretome are listed in Table 2. The most highly abundant cellular factors present in the HCMV secretome that contribute to WH/AG include the cytokines/chemokines (IL-6, osteoprotegerin, GRO, CCL3, CCL5, CCL7, CCL20, CXCL-5, and CXCL-16), receptors (TNF-RI & II, and ICAM-1), growth factors (TGF-β1 and HGF), ECM modifiers (MMP-1, TIMP-1, TIMP-2, TIMP-4) and the angiogenic RNase angiogenin. Interestingly, MCP-3 was induced over 85-fold in the HCMV secretome compared to the mock secretome. Most of the highly induced proteins detected in our assay (HCMV vs. mock) included chemokines such as CCL1, CCL3, CCL4, CCL5, CCL20, CXCL10, CXCL11). Interestingly, many of the genes that were identified by microarray analysis in the rat allograft hearts were also found in the HCMV secretome suggesting that the factors involved in HCMV-induced angiogenesis and wound healing are similar to those expressed in the RCMV-infected allografts and importantly, that these *in vitro* and *in vivo* processes are parallel to each other.

4.2 HCMV secretome induces angiogenesis in EC

Angiogenesis leading to vessel formation *in vivo* consists of a growth phase followed by a stabilization phase (Auerbach et al. 2003; Guidolin et al. 2004). Growth phase events include proteolytic digestion of the basement membrane (BM) and extracellular matrix (ECM) of the existing vessel, migration and proliferation of ECs, lumen formation within the EC sprout and anastomosis of sprouts to form neovessels. Stabilization involves arrest of EC proliferation, EC differentiation, intercellular adhesion and remodeling of the BM/ECM network to create an immature capillary. Key steps in both phases of angiogenesis can be modeled using an *in vitro* assay: the matrigel assay for capillary-like tubule formation (Wegener et al. 2000; Xiao et al. 2002). The extent of tubule formation and stabilization depends on factors produced by the EC themselves in coordination with exogenous angiogenic agonists or antagonists in the culture medium. We utilized the matrigel assay to test the angiogenic activity of the HCMV secretome. Low passage primary HUVEC were nutrient-starved in serum and growth factor-free endothelial SFM medium (SF-SFM) prior to harvest and resuspension in this same medium. Cells were introduced into 24-well trays containing polymerized plugs of growth-factor-reduced matrigel in the presence of 300μl of control and test supernatants. Control supernatants included SF-SFM and complete SFM containing 10% human serum and endothelial cell growth supplement. Each supernatant was tested in quadruplicate. Cell

phenotype was digitally recorded at 24 hrs to evaluate the degree of EC migration and differentiation into tubule structures, and again after two weeks to evaluate vessel survival and stability. Figure 1A graphically depicts two quantitative measures of angiogenesis: the number of enclosed polygonal spaces delimited by complete tubules (Lumens), and the number of nodes where branching tubules meet (Branch points). Figure 1B shows a representative example of each culture condition as a low power image. Figure 1C shows high power images to emphasize the differences in vessel integrity between conditions. Results with control supernatants confirmed that exogenous angiogenic factors are required to support the formation of a robust capillary network when EC are plated on GFR-matrigel. Specifically, EC cultured in complete SFM for 24 hrs aligned to form a meshwork of anastomosing tubules with multi-nodal branch points and enclosed lumens. In contrast, EC cultured in SF-SFM for 24 hrs were unable to form a consistent network of interconnecting tubules, with many cells generating incomplete tubes or aggregating in clumps. In contrast, EC cultured in the presence of the HCMV secretome supported the formation of an extensive polygonal capillary network. Vessels formed in the presence of the HCMV secretome appeared to be more defined than those formed in the presence of complete SFM, presumably reflecting increased stability of intercellular junctions. To test the degree of stabilization afforded by the HCMV supernatants, trays were kept in culture for 2 weeks (Figure 1D). By this time, networks induced by complete SFM had degenerated, but those induced by the HCMV secretome remained intact. Collectively, we show that the HCMV secretome contains factors that promote angiogenesis, and allow stabilization of neovessels and that generation of this active secretome requires HCMV replication (data not shown).

4.3 HCMV secretome induces wound healing in EC

For the WH assays we have used an electric cell-substrate impedance sensing (ECIS) system available from Applied Biophysics Inc., to monitor cell behavior. In ECIS, cells are grown on 8-well chamber slides with 250mm-diameter gold electrodes micro-fabricated onto each well bottom. A larger counter-electrode completes the circuit, using standard tissue culture medium as an electrolyte. When a weak AC signal is applied to the system, the presence of a confluent monolayer is reflected by a marked increase in impedance, since cells restrict the effective area available for current flow. Fluctuations in measured impedance occur in response to micro-motions of cells, and can be used as indications of cell viability or morphology change (Keese et al. 2004). ECIS technology has been adapted for wound healing assays (Charrier et al. 2005). Cells are grown to confluence on the arrays to achieve high impedance values and a transient voltage spike is applied to kill only the cells on the electrode. Normal ECIS measurements are then used to monitor of the rate of repopulation of the wounded area from cells surrounding the electrode, which is superior to traditional WH assays since it is entirely automated and allows continuous monitoring of the cellular response to wounding. We have used the ECIS system to measure the influence of the HCMV secretome on WH. For these assays, primary HUVEC were plated in two arrays in complete SFM and incubated overnight to allow establishment of a confluent monolayer. The next day, cells were serum starved prior to the addition of secretome samples from mock- and AD169- (plus or minus UV or foscarnet treatments) infected or HSV-1-infected HFs to duplicate wells. SF-SFM and complete SFM were used as negative and positive controls, respectively. Immediately after application of supernatants, arrays were placed in the electrode holders and impedance was measured for a few minutes. This confirmed the existence of confluent cell monolayers over each electrode and verified that the cells were healthy. All but one of the chambers of cells were wounded for 30s. As expected, the electrical wounding resulted in an immediate drop in impedance to the level of an open electrode. The subsequent rise in impedance due to migration and repopulation of the wound was monitored. As shown in Figure 2 under the presence of complete SFM, the wound was repopulated in about 6 hrs. Similarly, cells cultured in the presence of the HCMV secretome were able to repopulate the wound with the same kinetics as with complete SFM

although steady state resistance levels were slightly lower. In contrast, cells cultured in SF-SFM lacking growth factors, the mock secretome, or the secretomes from HCMV UV or Foscarnet treated cells all were unable to repopulate the electrode, even over the total 20 hrs measured. Similarly, cells incubated with a secretome derived from HSV-1 infected cells also failed to mediate WH, suggesting that the WH effects are specific for CMV. Importantly, these data clearly show that the HCMV secretome contains factors that promote cell migration into a mechanical wound. The ability of the HCMV secretome to mediate WH is due to active viral replication since both UV treated virions and foscarnet treated cells did not promote WH. Since foscarnet treatment of infected cells resulted in an inactive secretome, this observation suggests that a late kinetic class of HCMV gene(s) is involved in the generation of secretome WH factors. These observations correlate with studies in human heart transplant patients as well as our own observations in heart transplants in rats in which ganciclovir treatment prolongs graft survival.

5 Conclusions

There has been a steady progression to our understanding of role that HCMV plays during the acceleration of vascular diseases such as atherosclerosis, restenosis, and transplant vasculopathy associated with the development of chronic solid organ allograft rejection. This progress has been made through epidemiologic studies, the use of animal models of vascular disease and transplantation, as well as through the use of *in vitro* models that mimic the clinical scenario. While the precise mechanism(s) of action have yet to be fully elucidated, what has become clear over the last couple of decades of study is that HCMV is capable of modifying the extracellular host environment through the production and release of biologically active cellular factors including growth factors, cytokines, and ECM modifying enzymes. As we demonstrate here, the overall effect of this host manipulation is that the HCMV secretome is capable of mediating angiogenesis and wound healing, which are important processes that drive vascular disease formation. Indeed, many of the factors that we identified in the HCMV secretome were identified by microarray analysis in the rat allograft hearts suggesting that the factors involved in these two processes are similar. Future research meant to identify the specific up-stream gene targets that would make possible prevention or abrogation of the HCMV-associated vasculopathy and chronic allograft rejection will broaden the therapeutic profile used to combat this very important clinical problem.

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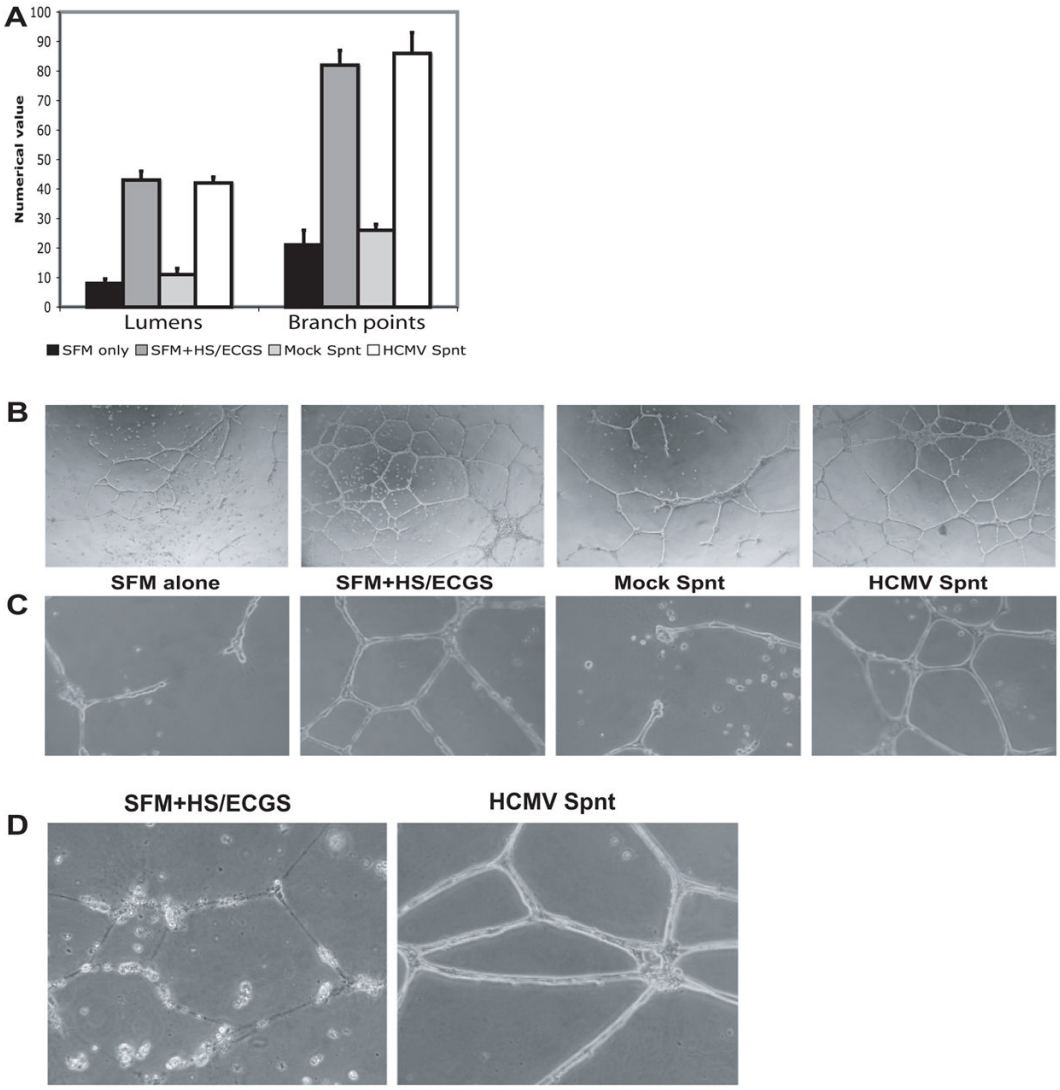

Figure 1. HCMV Secretome Mediates EC Tubule Formation

(A) Quantitation of two parameters of angiogenesis, lumen formation and number of branch points at 24 hours post plating on matrigel in the presence of control supernatants (SFM alone or SFM + HS/ECGS) and test supernatants conditioned by factors secreted by Mock- and HCMV-infected cells.

(B) Low power images of EC differentiation on matrigel.

(C) High power images, conditions as for (B), illustrating the integrity of individual tubules.

(D) Tubule survival after 2 weeks on matrigel in the presence of SFM plus HS/ECGS or supernatant conditioned by HCMV-infected cells.

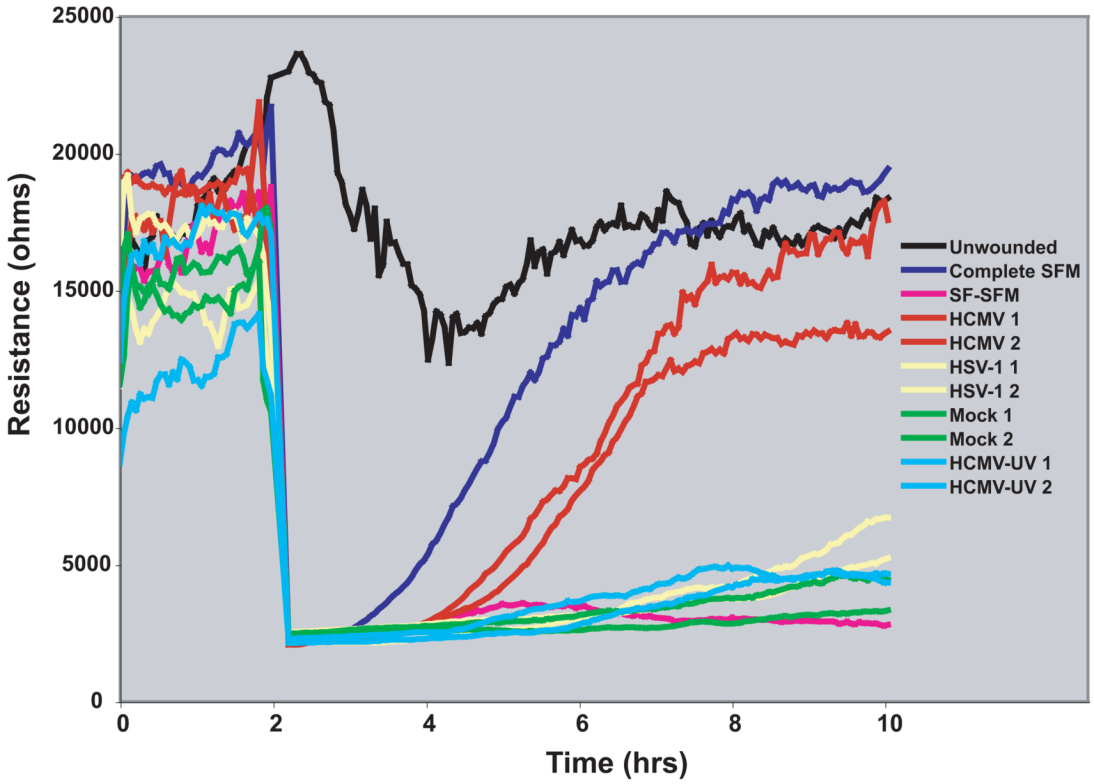

Figure 2. HCMV Secretome Mediates EC Wound Healing

Wound healing activity of the HCMV secretome. EC were grown to confluence on ECIS arrays and exposed to test supernatants prior to electrical wounding. Wound healing, as indicated by increasing resistance, is plotted as a function of time. Healing traces for duplicate wells are shown for the HSV-1 Secretome (yellow), mock (green), HCMV (red), or UV-inactivated HCMV (light blue). Controls traces include a negative control (SSFM; pink), a positive control (Complete SFM; dark blue) and an unwounded control (black). Cells exposed to the HCMV secretome show wound repair within 6–10 hrs, whereas cells exposed to the HSV-1 or mock secretomes repopulate the wound inefficiently, indicating that the production of wound healing factors is specific for HCMV infections.

Table 1

Cellular genes induced in infected vs. uninfected allografts at POD 21 and 28

		POD 21		POD28	
	Description	**Mock**	**RCMV**	**Mock**	**RCMV**
Pro-Angiogeneic	angiotensinogen	1.86	1.95	1.98	3.04*
	cathepsin B	1.76	2.38	1.70	3.45*
	cathepsin C	1.60	3.63	2.06	7.50*
	cathepsin E	6.62	27.26*	14.68	31.62*
	cathepsin S	5.54	12.86	7.40	19.31*
	cathepsin W	3.10	34.34*	11.11	30.74*
	cathepsin Y	13.04	58.28	26.13	76.60*
	chemokine (C-C motif) ligand 2	12.98	50.08*	40.79	69.13
	chemokine (C-C motif) ligand 4	11.90	66.04*	30.69	137.02
	chemokine (C-X-C motif) ligand 1	4.52	4.03	8.25	19.34
	chemokine (C-X-C motif) ligand 2	2.67	2.46	2.80	4.79
	chemokine (C-X-C motif) ligand 12	4.13	5.62	3.64	10.85*
	cysteine rich protein 61	27.47	37.53	22.21	19.56
	nephroblastoma overexpressed gene	11.39	9.65	4.02	8.11
	WNT1 inducible signaling pathway protein 2	34.70	5.24	5.68	15.03
	laminin, alpha 3	2.71	2.48	2.49	4.20*
	laminin receptor-like protein LAMRL5	17.31	81.01*	52.92	106.35
	fibronectin 1	3.82	3.01	2.38	12.11*
	secreted phosphoprotein 1	32.17	6.44	12.14	395.56*
	epidermal growth factor receptor	4.49	5.95*	5.15	10.81*
	fibroblast growth factor 17	5.13	5.83	5.46	4.45
	fibroblast growth factor receptor-like 1	2.64	2.90	2.60	3.77*
	FGF receptor activating protein 1	2.97	3.62	3.27	5.74*
	hepatocyte growth factor (scatter factor)	1.94	2.16	2.05	2.44
	hepatocyte growth factor activator	2.03	2.64*	2.56	3.29*
	hypoxia inducible factor 1, alpha subunit	2.75	5.24	4.00	8.37*

		POD 21		POD28	
	Description	**Mock**	**RCMV**	**Mock**	**RCMV**
	insulin 2	8.65	16.68	13.67	12.30
	insulin-like 6	2.29	2.39	2.35	2.51*
	insulin-like growth factor 2	2.57	2.46	2.09	2.87*
	IGF 2, binding protein 1	2.92	2.93	3.43	3.48
	IGF binding protein 5	3.34	3.16	2.84	3.05
	integrin alpha M	4.70	15.03*	7.80	34.54*
	integrin alpha X	3.13	4.92	3.70	6.19*
	integrin beta 7	7.16	17.27	10.24	19.29*
	interleukin 1 alpha	2.34	4.38*	4.21	4.96
	interleukin 1 beta	16.00	50.91	31.78	82.71*
	interleukin 6	1.42	2.13*	2.76	4.59
	midkine	3.94	3.27	2.62	2.33
	colony stimulating factor 1(macrophage)	12.42	10.84	11.33	10.81
	M-CSF I receptor	3.07	6.38	3.86	11.31*
	CSF 2 receptor, beta 1 (gran-mac)	3.27	5.21	4.14	6.59
	matrix metalloproteinase 12	17.23	4.17	8.46	367.09*
	matrix metalloproteinase 14	8.63	4.20	3.37	11.00*
	Matrix metalloproteinase 23	4.51	3.29	3.32	6.19*
	plasminogen activator inhibitor 2 type A	1.55	2.08	1.80	2.93*
	ser (or cys) proteinase inhibitor, member 1	8.88	10.34	15.03	20.39
	plasminogen activator, urokinase	6.62	12.91*	9.36	65.34*
	plasminogen activator, urokinase receptor	3.46	5.98	3.85	12.38*
	platelet-derived growth factor, C	2.36	2.48	2.47	3.89*
	transforming growth factor, beta 1	2.96	4.69	3.93	9.78*
	transforming growth factor, beta 2	6.57	10.85	13.96	4.69
	bone morphogenetic protein 2	4.24	5.78	5.00	6.82
	bone morphogenetic protein 7	3.57	3.89	3.89	3.73
	growth differentiation factor 15	3.03	2.83	4.62	5.17

		POD 21		POD28	
	Description	**Mock**	**RCMV**	**Mock**	**RCMV**
	lymphotoxin A	3.71	5.17*	6.28	5.10
	TNF superfamily member 6	3.96	11.71*	6.77	17.88*
	TNF superfamily member 11	2.50	3.66	2.97	3.92*
	TNF superfamily member 13	4.71	12.04*	7.05	17.88*
	TNF receptor superfamily member 1b	3.71	11.08*	6.53	13.83*
	TNF receptor superfamily member 4	3.47	8.75	6.13	10.48
	TNF receptor superfamily member 11b	3.96	18.64*	5.10	40.22*
	vascular endothelial growth factor D	3.33	3.33	3.26	3.18
	fms-related tyrosine kinase 4	3.99	3.56	3.75	3.92
Anti-Angiogeneic	interferon gamma	1.64	4.79*	4.33	10.78*
	interleukin 4 receptor	3.11	6.32*	3.69	6.02*
	interleukin 10	1.38	3.07*	2.11	3.86*
	interleukin 12b	3.65	5.35	4.54	3.58
	chemokine (C-X-C motif) ligand 10	28.49	222.50	143.15	178.07
	thrombospondin 2	5.95	2.93*	3.05	5.24
	tissue inhibitor of metalloproteinase 1	17.75	33.59	33.75	41.64
	tissue inhibitor of metalloproteinase 2	2.33	1.53	1.67	3.41*

*p<0.05 infected vs. uninfected.

Table 2

HCMV-induced secretome factors (Raybiotech Array Analysis).
Top-35 Most Abundant Factors in HCMV Secretome (Raybiotech Protein Array)

Protein	Mock Ave.	HCMV Ave.	HCMV/Mock Fold Change	Protein	Average Intensity
Angiopoietin 2	291	716	2.5	Growth related oncogene	58003
Receptor tyrosine kinase	1328	2931	2.2	Interleukin-6	57815
Fibroblast growth factor basic	417	768	1.8	Angiogenin	54454
Nerve growth factor beta	294	490	1.7	Matrix metalloproteinase 1, collagenase	51739
Cardiotropin-1	356	577	1.6	Hepatocyte growth factor	40025
CC chemokine, CCL28	163	488	3.0	Moncyte chemoattractant protein-3, CCL7	37093
Monocyte differentiation antigen CD14	1740	5366	3.1	Interferon induced protein-10, CXCL10	33507
Small inducible cytokine B16	3964	13384	3.4	Moncyte chemoattractant protein-1, CCL2	24258
Receptor tyrosine kinase	223	554	2.5	Metalloproteinase inhibitor 4	19538
CXC chemokine-5, ENA-78	1104	9583	8.7	Tumor necrosis factor receptor 1	19276
Fms-like tyrosine kinase-3 ligand	1637	3296	2.0	CC chemokine, CCL5, RANTES	18369
Granulocyte-macrophage growth factor	1185	6366	5.4	Osteoprotegerin	18227
CCchemokine-1, I-309	65	1589	24.3	Transforming growth factor beta-1	17923
Intercellular adhesion molecule- 1	3238	9342	2.9	Tumor necrosis factor receptor 2	13706
Intercellular adhesion molecule-3	180	622	3.5	Small inducible cytokine B16	13384
Interleukin-17	269	549	2.0	Macrophage inflammatory protein-1 alpha, CCL3	10631
Interleukin-2	344	637	1.9	Metalloproteinase inhibitor 2	9893
Interleukin-21 receptor	556	931	1.7	CXC chemokine, CXCL5	9583
Interleukin-6	5237	57815	11.0	Intercellular adhesion molecule-1	9342
Interleukin-6 receptor	946	1551	1.6	Metalloproteinase inhibitor 1	9329
Interleukin-7	1066	3596	3.4	Insulin-like growth factor binding protein-2	8953
CXC chemokine-8, IL-8	1265	4709	3.7	Macrophage inflammatory protein-3 alpha, CCL20	8672
CXC chemokine-10, IP-10	2019	33507	16.6	Granulocyte-macrophage growth factor	6366
CXCchemokine-11, ITAC	249	4872	19.6	Cell surface adhesion molecule	5874
Transforming growth factor beta-1	7220	17923	2.5	TNF receptor family member 6 (CD95)	5635
Hormone controlling metabolism	1142	1920	1.7	Monocyte differentiation antigen CD14 precursor	5366

Protein	Mock Ave.	HCMV Ave.	HCMV/Mock Fold Change	Protein	Average Intensity
Leukemia inhibitory factor	1309	2120	1.6	CXC chemokine, CXCL11	4872
CC chemokine 7, MCP-3	430	37093	86.2	Interleukin-8, CXC chemokine CXCL8	4709
CC chemokine-3, MIP-1α	694	10631	15.3	Interleukin-3	4478
CC chemokine-4, MIP-1β	295	3742	12.7	Insulin-like growth factor binding protein-4	4474
CC chemokine-20, MIP-3α	269	8672	32.3	Interleukine-1 alpha	4373
Neurotrophin 4, member of NGF family	214	574	2.7	Urokinase receptor	3772
Platelet-derived growth factor-AA	261	822	3.1	Macrophage colony growth factor	3761
Platelet-derived growth factor-BB	317	1097	3.5	Macrophage inflammatory protein-1 beta, CCL4	3742
CC chemokine-5, RANTES	1742	18369	10.5	Soluble glycoprotein 130, CD130	3673
CXC chemokine-12, SDF-1β	545	1013	1.9		
Sialic acid binding Ig-like lectin 5	281	453	1.6		
Tyrosine-protein kinase receptor	429	664	1.5		
Tumor Necrosis factor receptor 1	7243	19276	2.7		
Tumor Necrosis factor receptor 2	813	13706	16.9		
TNF-related abobtosis-inducing ligand	790	2007	2.5		