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Is HCMV a Tumor Promoter?

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Introduction / Overview

Increasing evidence implicates infectious agents as causal in human cancer. Despite decades of human genetic studies and searches for environmental carcinogens, only a very small percentage of major human malignancies are clearly attributable to known inborn genetic mutations or environmental carcinogens. Instead, an ever increasing percentage of human malignancies in the last several decades have been attributed to infectious agents, and vaccinations and other preventative measures are having a significant impact on infectious related cancer deaths from malignancies such as hepatoma, cervical cancer and gastric cancer (Demaria et al.).

In malignancies that are not currently attributable to infectious agents, chronic subacute inflammation plays a critical role in the transition from neoplastic precursor to full-blown invasive malignancy, and inflammation is considered the seventh hallmark of neoplasia (Balkwill et al., 2005; Colotta et al., 2009; Grivennikov et al.). This period of chronic inflammation may indeed be essential for the neoplastic process in malignancy, and may be facilitated by “promoters”. Cancer promoters are agents that may have no significant oncogenic impact on wild-type cells, but can drive preneoplastic cells toward full blown malignancy.

Infectious agents can be promoters of neoplastic transformation. For example, Hepatitis C virus chronically infects the liver and causes a persistent inflammatory immune response resulting in hepatoma (Berasain et al., 2009). Another example is Epstein Barr virus (EBV) in nasopharyngeal carcinoma. EBV is ubiquitous in the human population, and thus is not oncogenic under normal circumstances. However, EBV infection in the nasopharynx of individuals exposed to certain environmental carcinogens is critical in the development of nasopharyngeal carcinoma, through expression of latent EBV genes that promote cell growth and survival (Young and Rickinson, 2004).

HCMV: A viral promoter of malignancy?

Human cytomegalovirus (HCMV), as of this time, has not been clearly implicated in human cancer. Nevertheless, growing evidence that HCMV is specifically detected in a variety of human malignancies at low levels of expression raises the possibility that chronic infection caused by this herpesvirus could induce the same kind of “smoldering inflammation” seen with other pathogens associated with cancer. Many of the biological responses elicited by chronic HCMV infection are similar to those that support chronic inflammation, leukocyte dysfunction, angiogenesis and wound healing.

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What criteria might be used to determine whether HCMV plays a causal role in oncogenesis?

While investigators have postulated a role for HCMV in human neoplasia in the past, many of the early studies by Rapp and colleagues were not easily reproducible and lacked clear *in situ* histopathological correlations with the proposed diseases (Geder et al., 1977; Sanford et al., 1977). The concept of “hit and run” oncogenesis then arose to describe the possibility that HCMV oncogenic events might occur, after which the viral presence is unnecessary (Shen et al., 1997). Jindrich Cinatl's group has hypothesized for over a decade that HCMV might play an “oncomodulatory” role in the neoplastic process, and they have shown in multiple studies that HCMV infection can induce cellular responses that would provide a growth advantage for neoplastic cells (Cinatl et al., 2004) [5-7].

With the more recent identification by our group and others of HCMV infection specifically in preneoplastic and neoplastic tumor cells of malignant gliomas, prostate cancers and colorectal cancers, but not adjacent normal tissues, a greater urgency to answer the question of whether HCMV may play an etiological role in these malignancies has arisen (Cobbs et al., 2002; Harkins et al., 2002; Samanta et al., 2003). This case has been argued by Söderberg-Naucler and colleagues, who have confirmed the detection of HCMV at very low levels of expression in malignant glioma and several other malignancies, and who have proposed that the term HCMV “microinfection” be used to describe the very low level of infection found in cancer (Soderberg-Naucler, 2008).

Several studies, however, have failed to confirm the association of HCMV with glioma (Lau et al., 2005) (Poltermann et al., 2006) colon cancer (Akintola-Ogunremi, 2005 #1994), or other malignancies. In the study by Lau et al, 22 brain tumors of various histologic types and grades, four normal brains, six breast carcinomas, six colon carcinomas, six lung carcinomas, and six sarcomas were evaluated for the presence of CMV by polymerase chain reaction (PCR), *in situ* hybridization, and immunohistochemical methods (Lau et al., 2005). These authors did not detect CMV nucleic acid or protein in any of these specimens. It is important to note, however, that the aforementioned studies have not employed the highly sensitive immunohistochemical approach required for detection of endogenous, low level HCMV infection in human specimens. This discrepancy has been addressed in the study by Scheurer et al (Scheurer M, 2007; Scheurer et al., 2008)

Given the controversial nature of this novel concept, it is critical that a more clear understanding of the role of HCMV in tumor biology be obtained, since such knowledge may have profound implications with respect to potential therapies and preventive strategies.

Unfortunately, establishing causality for a ubiquitous virus that causes persistent infection in a majority of adults worldwide is difficult. Furthermore, Koch's postulates cannot be directly applied in the context of a potential oncogenic virus that may take years to induce or promote malignancy (Fredericks and Relman, 1996). For this reason modified criteria based on Hill's 9 criteria were developed by Fredericks and Relman to address this issue with modern molecular techniques, and are outlined below (Fredericks and Relman, 1996). For a potential infectious agent to be considered causal in human cancer, the authors proposed that the following criteria must be met:

1. Putative pathogen is present in most cases of disease
2. Only diseased tissue should harbor putative pathogen
3. Disease resolution should be accompanied by decreased genome of putative pathogen

4. Microbial sequences should be present before disease or correlate with disease severity
5. The nature of the microbial organism detected should be consistent with known biological characteristics of that group of organisms
6. Microbe-associated sequences detected in the diseased tissue should be corroborated at the cellular level
7. Molecular evidence should be reproducible

In this review, we will examine the oncogenic properties of HCMV, primarily focusing on published and unpublished work as it relates to malignant glioma. We suggest that an accumulating body of evidence implicates HCMV as a potential tumor promoter based on three general themes: 1) HCMV infection and expression is specifically detected in a high percentage of several human malignancies, 2) HCMV infection of non-neoplastic cells in a tumor (e.g., macrophages, fibroblasts, endothelial cells) likely contributes to the tumor microenvironment by induction of cytokines and growth factors, and 3) HCMV infection of tumor cells themselves likely leads to increased oncogenic potential through multiple tumor signaling pathways.

Background: Chronic Inflammation and Oncogenesis

Infectious agents that promote a chronic, low level of infection promote oncogenesis by induction of mutagenic chemical mediators, genetic variations in inflammatory cytokines, immune cell mediators, and by generally evoking an environment with characteristics of chronic wound healing (reviewed in (Balkwill et al., 2005; Grivennikov et al.)). Under such conditions, tumor associated macrophages (TAMs) play a pivotal role in both mediating inflammatory (M1) responses as well as immunosuppressive and growth (M2) responses (Allavena et al., 2008; Grivennikov et al., ; Tsujii et al., 1997; Tsujii et al., 1998). Such responses, which are critical in wound healing, also promote neoplastic growth. In response to cytokines such as IL-10 and TGF-beta, TAMs acquire M2 properties, promoting immune suppression and proliferation. In the case of malignant glioma, increased M2 macrophage infiltration in the tumor is associated with more aggressive tumor phenotype (Komohara et al., 2008). Inhibition of antitumor T-cell effector function also occurs in this environment, since IL-10 and TGF-beta can block dendritic cell (DC) maturation and attract regulatory T-cells (Tregs)(Gomez and Kruse, 2006). Tregs are potent inhibitors of the cell mediated T cell antitumor response(Gomez and Kruse, 2006).

An accumulating body of experimental evidence indicates that the chronic expression of TNF-alpha and IL-1 beta in the pre-malignant microenvironment can produce dramatic increases in the likelihood of malignant transformation via activation of the NF-kB transcriptional activator (Greten et al., 2004). Furthermore, two critical downstream effectors of this NF-kB pathway with respect to oncogenicity appear to be COX-2 and IL-6(Berasain et al., 2009; Fantini and Pallone, 2008; Karin, 2009). IL-6 induction and expression in tumor cells and tumor associated myeloid cells has an important role in chronic inflammatory oncogenic signaling, likely by activation of the STAT-3 transcriptional activator (Tanabe et al.). Hence, the NF-kB pathway has a dual effect in tumor promotion by preventing death of cells with malignant potential and by stimulating pro-inflammatory cytokines in infiltrating myeloid and lymphoid cells.

In malignant glioma, potent inflammatory pathways are present, which both increase the likelihood of reactive species involvement in mutagenesis, while simultaneously attracting microglia and macrophages into the local environment. Nevertheless, the host anti-tumor immune response in gliomas is highly ineffective (Gomez and Kruse, 2006; Parney et al.,

2000). Although infiltrating T-cells may be present, up to one third of cells in malignant gliomas express macrophage markers, and are thus likely to represent both intrinsic microglial cells from the CNS and infiltrating monocyte-derived cells (Gomez and Kruse, 2006; Hussain et al., 2006; Rossi et al., 1987). Those effector T-cells that are present in malignant gliomas are highly non-functional. Inactivation of infiltrating T-cells in gliomas may be due to the impact of T-cell anergy, which can occur when tumor associated immature DCs inactivate T-cells (Gomez and Kruse, 2006). Tregs, found at high levels in gliomas, also inhibit T-cell proliferation and activation (Humphries et al.).

TGF-beta, IL-10, and PGE-2 are key soluble mediators of immunosuppression in malignant glioma and other tumor types (Zou et al., 1999). TGF-beta and PGE-2 have both immunosuppressive and tumor proliferative properties (Tsujii et al., 1997; Tsujii et al., 1998; Williams et al., 1999). Upon binding to its receptor, COX-2 derived PGE-2 also promotes tumor cell migration, invasion, and angiogenesis (Tsujii et al., 1997; Tsujii et al., 1998). NF-kB is a critical mediator of the inflammatory cascade that leads to COX-2 induction, as well as the induction of iNOS and other inflammatory cytokines (IL-1beta, IL-6, and TNF-alpha). These cytokines, along with nitric oxide (NO) produced by TAMs and tumor cells via nitric oxide synthase (NOS) activation, are found at high levels in malignant gliomas and other tumors, and are important promoters of inflammation-driven oncogenesis and immunosuppression (Cobbs et al., 1995b; Hara and Okayasu, 2004; Jia et al.).

Our group became interested in the possible role of HCMV in cancer while studying the inflammatory pathways associated with grade IV malignant gliomas, or glioblastoma multiforme (GBM). We found that these tumors had high levels of iNOS expression and evidence of an elevated reactive oxygen and nitrogen environment (Cobbs et al., 1995a). Furthermore, we and others determined that GBMs expressed elevated levels of COX-2 (Hara and Okayasu, 2004; Shono et al., 2001). And, despite the fact that GBMs had evidence of increased macrophage/microglia infiltration and inflammation, patients with GBM were known to harbor profound systemic defects in cell mediated immunity and cytotoxic T cell function. Indeed, malignant gliomas are known to exist in a highly immunosuppressive microenvironment, characterized by high levels of expression of immunosuppressive agents such as TGF-beta, PGE-2, and IL-10 (Zou et al., 1999).

Because of the paradoxical inflammatory and immunosuppressive environment present in malignant gliomas, we speculated that a chronic viral infection in the tumor itself may be present. We hypothesized that HCMV might be able to persistently infect immature cells of glial origin, and cause a low level of chronic inflammation, while simultaneously promoting immune dysregulation. HCMV is a virus that can remain latent for life, and become reactivated in the setting of immunosuppression and / or inflammation (Britt, 2008). HCMV is tropic for fetal glial cells and is capable of persistently infecting GBM cells in vitro (Pari et al., 1998; Poland et al., 1990). HCMV also promotes a chronic inflammatory state associated with the increased expression of ROS, RNS and COX-2 (Harkins et al., 2002; Hsu et al., 2003; Maussang et al., 2009; Suzuki et al., 1997; Zhu et al., 2002). For these reasons we chose to look for evidence of HCMV infection in human GBM.

We developed highly sensitive immunohistochemical and in situ hybridization detection techniques for HCMV, and by using these techniques we have found that >95% of GBM specimens are infected with HCMV, and multiple HCMV gene products are expressed in the tumors. These findings have since been confirmed by several other groups of investigators (Mitchell et al., 2008; Scheurer et al., 2008; Soderberg-Naucler, 2008). Subsequently, using similar techniques, we determined that a high percentage of human prostate, colon and

breast cancers (unpublished data) are infected by HCMV (Harkins et al., 2002; Samanta et al., 2003).

A: The impact of HCMV infection on the tumor microenvironment

HCMV causes persistent infection of the bone marrow and other organ systems, and probably never exists in a true latent state (Reinke et al., 1999). Indeed, recurrent viral reactivation appears to result in a chronic immune response to the virus often characterized by a specific anti-HCMV T lymphocyte response of over 10% of circulating lymphocytes in immunocompetent individuals (Sylwester et al., 2005). HCMV reactivation in the myeloid population can occur in differentiating monocyte derived cells in the setting of inflammation and immunosuppression (Soderberg-Naucler et al., 1997; Taylor-Wiedeman et al., 1994). Conversely, HCMV infection of monocyte/macrophages leads to an induction of inflammatory mediators that can promote inflammation in the surrounding cells. This persistent HCMV infection of the monocyte/macrophage pool could cause paracrine secretion of inflammatory molecules that promote malignancy (Chan et al., 2008a).

HCMV could promote oncogenesis via infection of tumor associated macrophages (TAMs)

In human colorectal carcinomas and breast adenocarcinomas, we detected expression of the HCMV pp65 protein in infiltrating myeloid cells in the tumor microenvironment (Harkins et al., 2002). In malignant gliomas, we have also detected HCMV protein expression in cells with macrophage/microglial morphological features. Persistent HCMV infection and expression in TAMs could have profound effects on tumor biology since it has been established that HCMV can persistently infect monocyte/macrophage lineage cells without cytopathic effects (Ibanez et al., 1991; Jarvis and Nelson, 2002a; Jarvis and Nelson, 2002b; Riegler et al., 2000; Sinclair and Sissons, 1996; Sinzger et al., 1996). TAMs are a major component of the tumor microenvironment and are attracted to hypoxic areas of tumors due to inflammatory cytokines and HIF-1 dependent upregulation of molecules such as SDF-1 and VEGF (Lazennec and Richmond, ; Oh et al., 2001).

HCMV appears to target monocytes *in vivo*, and once infected, HCMV viral survival benefits from induction of a strong inflammatory response in these monocyte/macrophages (Sinclair and Sissons, 1996). Recently, Chan et al demonstrated that the HCMV-infected monocyte transcriptome displayed a unique M1/M2 polarization signature that was skewed toward the classical M1 inflammatory phenotype, while still having components of the immunosuppressive M2 phenotype (Chan et al., 2008b). Furthermore, induction of NF- κ B and PI(3)K pathways following HCMV infection was required for the initiation of monocyte-to-macrophage differentiation (Chan et al., 2009). HCMV infection of monocyte/macrophages was associated with an induction of cytokines IL-1, IL-6 and TNF- α , which can contribute to the classic proinflammatory macrophage/microglial phenotype and promote viral replication. Indeed, HCMV induced a 280 fold induction of IL-6 expression, and a 15 fold induction of TNF- α in infected macrophages (Chan et al., 2008b). These cytokines would be expected to produce an oncogenic microenvironment since chronic expression of TNF- α and IL-6 are directly linked to oncogenic transformation in inflammation-induced animal models of cancer (Grivennikov et al.).

While these cytokines are prototypical of the M1 type of inflammatory cascade, HCMV infection of macrophages simultaneously results in the induction of M2 type macrophage responses, by inducing IL-10, IL-1 β , and IL-18 responses (Chan et al., 2008b). Thus, HCMV infection of TAMs promotes both an M1 and M2 “schizophrenic” phenotype of

macrophage expression in the tumor microenvironment, which has been found to promote neoplastic progression (Allavena et al., 2008).

HCMV could promote oncogenesis through factors secreted by fibroblasts and other cells in the tumor microenvironment

Studies of the role of HCMV in transplant vascular sclerosis have important implications for the potential role of HCMV in human malignancy. This body of work reinforces the concept that chronic HCMV infection of non-neoplastic cells can have major paracrine effects on adjacent cells in the microenvironment. In a provocative work by Jay Nelson's group, the "secretome" induced specifically by HCMV infection of dermal fibroblasts was found to contain exceedingly high levels of multiple wound healing associated growth factors, matrix remodeling proteins, and angiogenic factors (Dumortier et al., 2008; Streblow et al., 2008). Among the factors highly secreted by HCMV infected cells, were members of the TGF-beta signaling pathway and extracellular matrix remodeling enzymes (e.g., MMPs). These proteins facilitate immunosuppression, wound healing and angiogenesis by remodeling of the extracellular matrix (ECM) and activating latent growth factors.

In our studies of HCMV infection in colon, prostate and breast cancer, we identified scattered areas of stromal fibroblasts infected with HCMV (Harkins et al., 2002; Samanta et al., 2003). If fibroblasts within the tumor microenvironment are infected and persistently secrete factors due to HCMV infection, this could significantly influence the growth of the tumor. The work by Dumortier et al. revealed that cytokines and chemokines including IL-6, GM-CSF, MIP-1 alpha, and MCP-1 were among the most abundant wound healing and angiogenic factors induced by HCMV infection (Dumortier et al., 2008). These paracrine-secreted factors induced robust wound healing and angiogenesis in model systems.

In a tumor microenvironment *in vivo*, these same chemokines would be expected to promote infiltration of macrophages, mitogenic activity, angiogenesis and activation of downstream inflammatory cascades. Mitogenic activity would be stimulated by the growth factors that are secreted by HCMV-infected fibroblasts at high levels such as GDNF, PDGF-AA, -AB, and -BB, TGFbeta1, and hepatocyte growth factor (HGF). Strikingly, GDNF secretion was induced 225-fold by HCMV infected fibroblasts. GDNF secretion would likely provide a strong oncogenic stimulus in the setting of a tumor microenvironment since GDNF is a potent promoter of cell survival and can confer mitogenic activity and chemoresistance on glioblastoma cells and proliferation, survival, cell migration and anchorage independent growth in breast cancer cells (Esseghir et al., 2007; Ng et al., 2009).

The PDGF growth factors are also very strong mitogens implicated in oncogenesis and progression of multiple malignancies including glioblastoma and breast cancer (Ahmad et al., Campbell et al., 2005; Shih and Holland, 2006). High levels of circulating PDGFs could markedly promote tumor progression if secreted into the tumor microenvironment. In addition to growth factors, high levels of many extracellular matrix modifiers (including MMP-1, TIMP-1, TIMP-2, uPAR) were secreted by HCMV infected cells. These ECM modulators are critical for both wound healing and tumor invasion and metastasis.

Bone marrow derived mesenchymal stem cells (BM-MSC), if infected by HCMV, could also play a significant role in promoting the oncogenic phenotype. Increasingly, the critical role of BM-MSCs in malignancy has become appreciated. These cells can regulate the differentiation and proliferation of adjacent hematopoietic precursor cells and contribute to the regeneration of mesenchymal tissues, including bone, cartilage, fat and connective tissue, and notably malignant tissues (Roorda et al., 2009). BM-MSCs are permissive to productive HCMV infection and HCMV dysregulates the function of MSCs by altering their

role in vasculogenesis and differentiation of surrounding cells (Smirnov et al., 2007). During tumor development, BM-MSCs are attracted to and infiltrate areas of neoplasia and play a critical role in the development of tumor neovasculature (Roorda et al., 2009). Indeed, these cells are essential for tumor growth beyond an avascular threshold. If BM-MSCs are infected with HCMV, they are likely to deliver virus directly to the tumor and lead to infection of tumor cells, as has been demonstrated experimentally in human gliomas (Yong et al., 2009). Infiltration of a growing tumor by HCMV-infected BM-MSCs may thus be a primary route of virus access the tumor.

HCMV promotes immune evasion of infected cells and could contribute to an immuno-privileged tumor microenvironment

A critical component in inflammation-associated malignancies is the loss of normal anti-tumor immune function in the tumor microenvironment. In addition to the tumor promoting effects of HCMV infection of monocyte /macrophages, expression of HCMV gene products by infected tumor cells could dramatically alter the host's ability to recognize tumor cells. A variety of tumor-derived factors contribute to the emergence of complex local and regional immunosuppressive networks, including VEGF, IL-10, TGF-beta, and PGE-2 (Kim et al., 2006a; Kim et al., 2006b). Cytotoxic T cell (CD8+) and NK cell responses are critical effectors of normal host anti-tumor immunosurveillance.

Through millions of years of co-evolution with the host, HCMV has evolved multiple strategies to allow persistent viral infection through a complex array of immune evasion strategies (Hengel et al., 1998; Loenen et al., 2001; Michelson, 1999; Scholz et al., 2003; Wiertz et al., 1997). Several HCMV gene products are expressed as immediate early and early viral genes to block the host cell MHC class I antigen expression, which is required for CD8+ cytotoxic tumor killing. The UL83 gene product pp65, which we and others have consistently detected in malignant glioma tumor cells, blocks antigen presentation of IE1-72, one of the earliest immunodominant HCMV epitopes, from CD8+ T cells. To further mask CD8+ recognition of virus-infected cells, the HCMV US3 gene product causes retention of MHC class I complexes in the ER (Greijer et al., 2001). The US11 gene product causes dislocation of the MHC class I heavy chain into the cytoplasm and the US2 gene product causes export of the MHC class I heavy chain from the ER (Benz and Hengel, 2000; Besold et al., 2009). The US6 gene product causes inhibition of the TAP-mediated peptide translocation into the ER.

NK cell function is also likely incapacitated by HCMV infection of tumor cells. Since downregulation of MHC class I antigen is a mechanism by which NK cells target foreign or "non self" cells for elimination, HCMV has evolved a unique strategy to circumvent its own virally-mediated downregulation of MHC class I antigen to subvert NK cell recognition of infected cells which have downregulated cell surface levels of MHC class I antigen. To accomplish this, HCMV expresses the UL18 glycoprotein, a MHC class I homologue, thereby avoiding killing by NK cells (Farrell et al., 1997).

Through a complex interaction with tumor cells and TAMs, HCMV infection is likely to severely impair function of tumor antigen presentation by dendritic cells (DCs) in the tumor microenvironment. Interleukin-10 (IL-10) suppresses the maturation and cytokine production of dendritic cells (DCs), key regulators of adaptive immunity, and prevents the activation and polarization of naive T cells towards protective gamma interferon-producing effectors. Treatment of immature DCs (iDCs) with supernatant from HCMV-infected cultures inhibited both the lipopolysaccharide-induced DC maturation and proinflammatory cytokine production (Chang et al., 2004). The HCMV viral IL-10 can also interfere with DC,

microglial and macrophage function through interference with normal differentiation and cytokine production (Cheeran et al., 2003; Nachtwey and Spencer, 2008).

In addition to direct immunomodulatory effects on myeloid cells within the tumor, expression of immunosuppressive cytokines by HCMV infected tumor cells, tumor infiltrating macrophages and fibroblasts would provide a virtually impenetrable environment for the host anti-tumor immune system. As previously noted, TGF-beta, IL-10 and PGE-2 are among the most potent immunosuppressive cytokines. These cytokines are all induced by HCMV infected cells, and in combination would be expected to have a profound immunosuppressive effect on the host antitumor response (Maussang et al., 2009; Wang et al., 2006; Zhu et al., 2002).

B. The oncomodulatory impact of HCMV infection on pre-neoplastic and neoplastic cells

While the data presented above indicate that HCMV has the potential to contribute to oncogenesis by expressing gene products in cells of the tumor microenvironment, myriad other data indicate that HCMV can have direct oncogenic effects on tumor cells and tumor precursor cells themselves. As the field of cancer research has evolved, an increased appreciation of the role of adult stem cells and cancer stem cells in the neoplastic process has emerged. An agent that could impact the differentiation state and self renewal capacity of stem cells, while simultaneously causing DNA mutations, activation of growth signaling pathways and inhibition of tumor suppressor pathways, would be an ideal agent for promoting oncogenesis. We will discuss below the evidence, primarily as it relates to malignant glioma oncogenesis, that HCMV has the potential to exert direct oncogenic effects on tumor initiating stem cells and tumor cells themselves.

HCMV infects stem cells, impacts cellular differentiation and contributes to initial oncogenic events

HCMV is a neurotropic virus which can specifically infect neural precursor cells and impact neuronal differentiation. Recent data (Luo et al.) demonstrated that HCMV can induce premature differentiation of both neurons and astrocytes, which might explain in part the severe developmental delays documented in cases of neonatal (congenital) HCMV infection. HCMV can also persistently infect and reside latently in neural precursor cells and may be reactivated under specific circumstances. Reactivation of CMV during neural precursor differentiation has been studied in mouse models. Experiments using both human and murine CMV indicate that upon reactivation, induction of the viral immediate early (IE) genes occurs, and subsequent viral gene expression is activated, during differentiation of NPCs (Cheeran et al., 2005; Matsukage et al., 2006; Odeberg et al., 2006). In transgenic mice, IE activity occurs specifically in proliferating (BrdU+) cells in the subventricular zone (SVZ) of the brain, suggesting that this stem cell population is the most susceptible site for activation of the CMV IE promoter (Bakker et al., 2004). In studies of adult mice infected *in utero* with murine CMV, IE gene expression could be detected in nestin(+), GFAP(+), cells in the SVZ later in life, suggesting that either partial viral gene expression or full infectious viral life cycle occurs in these neuro-glial stem cells throughout adult life (Shinmura et al., 1997). Similarities between murine and human CMV indicate that HCMV may also persistently infect the NPC population in the adult human brain. Indeed, human NPCs are fully permissive to HCMV infection as these cells differentiate either along neuronal or astroglial lineages (Luo et al., 2008). A clinical isolate of HCMV (TR) showed significantly decreased cytopathic effect in comparison with the Towne laboratory strain in these cells suggesting that NPCs may remain persistently infected *in vivo* (Luo et al.).

Recent evidence suggests that a subpopulation of cancers cells, termed cancer stem cells, is responsible for tumor initiation, resistance to therapy and recurrence in several malignancies, including gliomas, breast, colon, prostate cancer, leukemia, and others (Wang). The cell surface protein CD133 has proven useful in the enrichment of normal neural stem cells. The concept of glioma stem cells is based on the striking similarities observed between the self-renewal capacity of stem cells and that of a CD133 positive subpopulation of cancer cells freshly isolated from GBM tissues. The self-renewal capacity of NSC ensures their longevity, while also rendering these cells susceptible to accumulation of multiple genetic mutations culminating with the malignant transformation and formation of a primary brain tumor. The ability of CD133 positive glioma-derived cells to form tumors in nude mice (Singh et al., 2003; Singh et al., 2004) supports the hypothesis that this cell subpopulation may indeed comprise the “tumor-initiating cells”. Recent studies have shown that the CD133 positive fraction of tumor cells contributes to glioma radio-resistance (Bao et al., 2006). Since HCMV latently resides in stem cells of the bone marrow (Goodrum et al., 2002), we investigated whether HCMV gene products are present within the “stem-like”, CD133+, glioma-derived cultures. Primary GBM tissue obtained from patients undergoing surgical resection was subjected to enzymatic digest and cells were further sorted for CD133 using the Myltenyi CD133 magnetic beads coated antibody. Our data indicate that HCMV IE1 is preferentially expressed in the CD133+ glioma subpopulation (while largely absent in the CD133-tumor cells, data not shown), suggesting that HCMV may be selectively reactivated in and modulate the oncogenic phenotype of this tumor cell subpopulation.

In situ double immunofluorescence detection of CD133 and IE1 in GBM frozen tissue sections showed that CD133+ glioma cells express HCMV IE1 in a majority of the gliomas we have sampled to date, with HCMV (IE1) expression occurring in about 40-60% of the CD133 positive cells (not shown). Identification of the essential HCMV gene product IE1 in glioma “stem-like” cells *in situ* constitutes evidence that CD133+ positive cells may be *persistently* infected with HCMV in glioma patients. Ongoing studies in our laboratory are investigating how HCMV modulates differentiation and self renewal of glioma stem like cells. Preliminary data suggest that HCMV infection and expression of certain viral proteins increase the expression of stem cell markers (e.g., Sox 2, Oct 3-4, E-cadherin) and promote self-renewal of glioma stem cells as measured by sphere formation assays and induction of Bmi1.

HCMV causes DNA mutations and dysregulates cellular DNA repair mechanisms

In the setting of persistent infection of vulnerable adult stem cells, HCMV-mediated genomic injury could promote oncogenesis. Indeed, the HCMV IE1 and IE2 gene products are mutagenic (Shen et al., 1997). When combined with other viral oncogenic proteins that release cell cycle checkpoint controls, such as the adenovirus E1A protein, the IE1-72 and IE2-86 HCMV gene products are able to induce oncogenic transformation of cells (Shen et al., 1997). In addition to dysregulating the cell cycle, IE1-72 and IE2-86 gene products block TNF- α mediated apoptosis and are mutagenic (Zhu et al., 1995). Several studies demonstrated that HCMV induces chromosomal aberrations (Albrecht et al., 2004; Siew et al., 2009). Specifically the viral protein UL76 has been shown to induce micronuclei, misaligned chromosomes, lagging and bridging (Siew et al., 2009). Infection with HCMV of human cells in the S phase of the cell cycle results in specific chromosome breaks at various band positions on chromosome 1 (Fortunato et al., 2000; Fortunato and Spector, 2003), including 1q21 and 1q42 (Nystad et al., 2008). These HCMV effects on chromosomal integrity and the synergistic activity of HCMV infection in combination with other cytotoxic agents (Albrecht et al., 2004) contributes significantly to global genetic instability, which is a major cancer promoter. Furthermore, HCMV has been shown to disrupt DNA repair

pathways, including the activity of ATM and ATR (Luo et al., 2007). Recent evidence indicates that the HCMV IE1 gene product can cause sustained hTERT telomerase activity in glioblastoma cells, and that hTERT expression co-localizes with IE1 detection in human tumors *in vivo* (Straat et al., 2009).

HCMV modulates cell cycle via interaction with tumor suppressor proteins P53 and RB

In addition to their mutagenic and anti-apoptotic properties, the HCMV IE gene products (IE1-72 and IE2-86) can dysregulate cell cycle checkpoint controls by interacting with the p53 and Rb tumor suppressor proteins. In some cell types, IE1-72 and IE2-86 can mediate a growth-arrest in G1 in infected cells to allow viral DNA replication at the expense of host replication. In other cell types, IE1-72 and IE2-86 can alter the cell cycle distribution towards S and G2/M phases to generate an environment conducive to proliferation (Castillo et al., 2005; Castillo and Kowalik, 2002). IE2-86, but not IE1-72, can induce quiescent cells into S phase and delay cell cycle exit, and in the presence of a non-functioning p53 tumor suppressor protein, IE1-72 induces S phase and delays cell cycle exit.

We have shown that the HCMV IE1-72 gene product has the potential to promote glioblastoma cell proliferation by dysregulating the key tumor pathways. We determined that sustained IE1 expression can suppress p53 and Rb tumor suppressor activity, while simultaneously driving the PI3K/Akt oncogenic signaling pathway in U87 and U118 glioblastoma cells. We also found that this effect may depend on the cellular state of differentiation and/or immortalization, such that viral gene expression may play a paradoxical role - promoting growth in tumor cells while blocking growth in non-transformed astrocytes or fibroblasts (Cobbs et al., 2007; Cobbs et al., 2008). Our published studies demonstrate that IE1 stable expression led to increased cell proliferation in the U87 GBM cell line (40% increase over control transfected cells (Cobbs et al., 2008)). We subsequently investigated the pathways controlling cell proliferation, survival and cell cycle progression. We found that IE1 stable expression in the U87 glioma cells induced p-Akt, inhibited Rb function by promoting its phosphorylation, and decreased the p53 family of proteins expression levels (Cobbs et al., 2008). Taken together, these data indicate that HCMV IE1 gene product can act as a viral oncogene and modulate proliferation, survival, and cell cycle progression of human glioblastoma cells. Another HCMV protein, UL69, is required for the HCMV-mediated G1 arrest seen in HCMV infected cells (Hayashi et al., 2000). HCMV deletion mutants lacking UL69 are unable to efficiently induce G1 arrest in infected cells (Hayashi et al., 2000). Thus it is conceivable that a mutant HCMV virus that expresses IE proteins in the absence of UL69 could promote unabated cell cycle progression and mutagenesis. In addition, HCMV expresses two proteins, pp71 and UL97 that can phosphorylate and inactivate the Rb family of tumor suppressor proteins (Hume et al., 2008; Kalejta et al., 2003). The UL97 protein causes constitutive phosphorylation of Rb, which is not able to be suppressed by normal cellular cyclin dependent kinase inhibitor proteins such as p21/Waf1. Our published data show that overexpression of HCMV IE1 in primary GBM-derived cells promotes cell cycle progression and BrdU incorporation. This indicates that upon reactivation, HCMV IE1 could modulate critical oncogenic features of glioma cells, including cell proliferation and survival (Cobbs et al., 2008). Unpublished data from our laboratory demonstrate that human malignant gliomas express HCMV pp71 *in situ* (not shown).

HCMV US28 encodes a viral “oncoprotein”

The US28 chemokine receptor encoded by HCMV is bona fide viral oncoprotein (Maussang et al., 2006). It binds a broad spectrum of chemokines, including SDF-1, CCL2/MCP-1,

CCL5/RANTES, and CX3CL1/Fractalkine, and, unlike its cellular homologue CCR1, US28 exhibits constitutive activity (Gao and Murphy, 1994). Ectopic expression of US28 induced a pro-angiogenic and transformed phenotype *in vivo*, by up-regulating vascular endothelial growth factor (VEGF) (Maussang et al., 2006). The induction of VEGF expression as a result of HCMV infection in U373 glioblastoma cells was due to the constitutive activation of US28, since a US28-deficient mutant HCMV did not induce VEGF (Maussang et al., 2006). Thus, the angiogenic (i.e., aggressive) phenotype in some tumors that express US28 might be due to the oncogenic properties of this gene acting in concert with other viral proteins, such as the immediate early (IE1 and IE2) gene products discussed above, to facilitate tumor initiation and progression following infection. Most recently, studies by Slinger et al have shown that HCMV US28 promotes a paracrine oncogenic signaling loop, by inducing activation of the IL6-phospho-Stat3 axis, which has been associated with poor outcome in malignant gliomas (Slinger et al.). Consistent with these data, our preliminary microarray and Taqman analyses indicate that both IE1 and US28 are highly expressed in patient - derived GBM biopsy specimens. In addition to its ability to induce oncogenic transformation and promote angiogenesis, the HCMV encoded chemokine receptor US28 promotes cell migration toward chemokines RANTES and MCP-1 (Streblow et al., 1999), which are abundantly expressed in malignant gliomas (Desbaillets et al., 1994).

HCMV infection activates cellular oncogenes and inhibits cancer cell apoptosis

In normal human fibroblasts, HCMV infection activates cellular proto-oncogenes, cyclins and kinases involved in cell division and cell survival pathways including *c-myc*, *c-fos*, *c-jun*, cyclin-B, cyclin-E, MAPK, ERK 1/2, and PI3-kinase (Chen et al., 2001; Hagemeier et al., 1992; Johnson et al., 2000; Johnson et al., 2001) (Jault et al., 1995). HCMV can also induce transcription factors such as NF- κ B that activate cell survival pathways in normal and tumor cells (Yurochko et al., 1995) and provide positive feedback for further HCMV immediate early gene transcription. In persistently infected neuroblastoma cells, HCMV induces the anti-apoptotic protein Bcl-2 resulting in acquired resistance to cytotoxic drugs such as cisplatin and etoposide, which can be reversed after treating the cells with the antiviral drug ganciclovir (GCV) (Cinatl et al., 1998). Several HCMV proteins have distinct anti-apoptotic activity, such as HCMV UL36 which binds to the prodomain of caspase-8 inhibiting Fas-mediated apoptosis and HCMV UL37, which inhibits activity of pro-apoptotic proteins Bax and Bak (reviewed in (Michaelis et al., 2009)). Studies using p73 knock out central nervous cells showed that the p53 gene product homologue (ie, p73) mediates HCMV brain tropism and promotes resistance to apoptosis in HCMV infected brain cells, thus providing a molecular mechanism for HCMV-induced developmental neural defects (Allart et al., 2002). Studies using PC3 prostate cancer cells as a model system have shown that HCMV infection or ectopic expression of HCMV IE1/IE2 induced increased invasion of these transformed cells by upregulating surface levels of integrins (β 1) and activation of *c-Myc* and FAK. HCMV infection also enhanced the attachment of prostate cancer cells to endothelial cells and transendothelial cell migration (Blaheta et al., 2006). Studies conducted in neuroblastoma cells demonstrated that HCMV infection modulates expression levels of several oncogenes and tumor suppressor proteins (Cinatl et al., 1996). Using the same cancer cell model, the authors found that HCMV modulates NCAM levels to neuroblastoma cell dissemination (Blaheta et al., 2004).

HCMV activation of cellular RTKs

To directly examine the possibility that HCMV can initiate oncogenic signaling, we conducted experiments to determine whether HCMV infection and gene expression could modulate key signaling pathways in human cells, including immortalized astrocytes and

glioblastoma cells. Using whole virus, we found that short term stimulation with HCMV activates Akt, PLC γ and FAK signaling. Since we detected phosphorylation of a likely receptor tyrosine kinase (RTK), distinct from EGFR (Cobbs et al., 2007; Isaacson et al., 2007) and engaging Akt, PLC γ and FAK, we explored the possibility that another RTK might be activated by HCMV. We utilized a human phospho-RTK array as a screening tool to detect the relative phosphorylation of 42 different human RTKs. We discovered that PDGFR α phosphorylation is dramatically induced in response to short term HCMV infection (Soroceanu et al., 2008).

These data suggest that HCMV may initiate oncogenic signaling in these tumors by engaging this tyrosine kinase receptor. To ascertain specificity of the HCMV-induced PDGFR α phosphorylation, we used siRNA technology to knock down the PDGFR α in human fibroblast cells and tested whether they are susceptible to HCMV infection. We determined that in the absence of a functional PDGFR α we could not detect IE1 immediate early gene product 12h following HCMV infection of PDGFR KD cells, while the control siRNA treated cells were readily infected. This suggests that PDGFR α is a necessary cellular receptor for HCMV infection and IE1 gene expression (Soroceanu et al., 2008). We next tested the ability of currently available PDGFR α blocking reagents to inhibit HCMV entry, gene expression, and virus production. Our data showed that using either the IMC-3G3 PDGFR α blocking antibody or the small molecule Gleevec (which inhibits receptor tyrosine kinase function) greatly inhibited HCMV gene expression and downstream HCMV-induced oncogenic signaling, such as the PI3K-Akt pathway (Soroceanu et al., 2008). Other important modulators of angiogenesis and tumor progression, the trombospodins (TSP1-2), were shown to be downregulated by HCMV in both human glioma cells (Cinatl et al., 1999a) as well as in human retinal glial cells, where HCMV inhibited expression of both TSP1 and TSP2 (Cinatl et al., 2000).

The PDGFR α -receptor gene is amplified in a subset (13%) of human glioblastoma (2008) suggesting that activation of PDGF receptor signaling confers a selective growth advantage in tumor growth. Evidence from mouse models of gliomas suggests that genetic alterations, such as loss of *Ink4Arf* locus in cooperation with exacerbated growth factor/growth factor receptor signaling in neural precursor cells can drive gliomagenesis (Holland, 2001). *In vivo* gene transfer of PDGF to neural precursor cells and astrocytes induces the formation of high grade gliomas in a dose dependent manner (Shih and Holland, 2006), suggesting that chronic activation of the PDGF receptors can promote proliferation of glial precursors and furthermore activate downstream signaling pathways (such as the PI3K-Akt) sufficient to drive tumorigenesis. Recent experimental evidence revealed that adult neural precursors driven to express high levels of PDGF had the capacity to give rise to gliomas and recruit resident progenitor cells in the mitogenic environment of the tumor (Assanah et al., 2006). A subpopulation of the neural progenitor cells located in the subventricular zone and positive for PDGFR α have been shown to have neural stem cell characteristics and respond to increased PDGF signaling by generating “glioma-like” lesions in the adult brain (Erlandsson et al., 2006). Chronic activation of the receptor in the SVZ B cells resulted in blocked differentiation, increased proliferation and generation of hyperplastic lesions, thus supporting the notion that this sub-population of cells may serve the role of tumor-initiating stem cell (Jackson et al., 2006). Unpublished data from our laboratory demonstrates co-localization of HCMV IE1 and human PDGFR α as well as HCMV glycoprotein B and hPDGFR α *in situ* in human GBM (immunohistochemical and RT-PCR analyses). Taken together these data suggest that a stimulus capable of inducing chronic activation of PDGFR α in glial precursors – such as HCMV-is likely involved in gliomagenesis.

HCMV glycoprotein-B is the most abundant and highly antigenic viral envelope protein. Our published data shows that HCMV gB directly binds and activates the human PDGFR

alpha (Soroceanu et al., 2008), which is amplified and over-expressed in several human malignancies, including gliomas. Endogenous expression of HCMV gB has been documented in human gliomas ((Mitchell, 2008) and our unpublished results). Recent data from our laboratory demonstrate that ectopic expression of HCMV gB promotes glioma cell migration, by inducing sustained PDGFR α phosphorylation and activation of downstream signaling pathways, including phosphor-AKT, FAK, Src (not shown). These results suggest that HCMV infected tumor cells may signal onto neighboring tumor and endothelial cells and thus sustain autocrine and paracrine signaling which promote tumor cell migration and angiogenesis. Increased invasion and angiogenesis are hallmarks of advanced neoplastic disease, suggesting that HCMV infection may promote a shift towards a more malignant tumor phenotype, at least in human gliomas. Below we discuss some specific mechanisms through which HCMV modulates tumor cell invasion and angiogenesis.

Mechanisms of angiogenesis and tumor cell invasion: Evidence for the involvement of HCMV

Human brain capillary endothelial cells are permissive for infection by HCMV and infection of endothelial cells by HCMV results in cytoplasmic p53 sequestration and escape from apoptosis (Kovacs et al., 1996). The HCMV IE1-72 and IE2-86 proteins increase vascular smooth muscle cell migration, proliferation, and expression of PDGF- β -receptor (Reinhardt et al., 2005) and IE2-86 can promote endothelial proliferation by binding and inactivating p53 in endothelial cells (Kovacs et al., 1996). Expression of thrombospondin-1 (TSP-1), a potent inhibitor of angiogenesis in gliomas (Hsu et al., 1996), is suppressed after HCMV infection of human fibroblasts and glioma cells (Cinatl et al., 1999b; Zhu et al., 1998). Inhibition of TSP-1 expression in glioma cells promotes glioma angiogenesis *in vivo* and is associated with a more malignant glioma phenotype (Hsu et al., 2003; Tenan et al., 2000). HCMV-mediated activation of COX-2 may also promote angiogenesis in tumor cells since COX-2 induces the expression of VEGF, bFGF, PDGF, iNOS, and TGF- α in tumor cells and promotes capillary endothelial cell migration and tube formation (Tsujii et al., 1998). Furthermore, as stated, HCMV US28 constitutive activity induces an angiogenic phenotype in human glioma cell lines (Maussang et al., 2006).

HCMV promotes glioma cell invasiveness by engaging PDGFR α and the α v β 3 integrin. We have previously demonstrated that HCMV can promote glioma cell invasiveness (Cobbs et al., 2007). Given the documented role of integrins and the PDGFRs in mediating glioma cell motility (Ding et al., 2003), we have performed experiments to investigate the role of integrins and PDGFR α in HCMV-induced cell motility. Scrape-wound assays showed that both α v β 3 and PDGFR α blocking antibodies inhibited HCMV-induced cell migration (not shown). Double immunofluorescence analyses showed that HCMV can induce α v β 3 recruitment to the focal adhesions to the same extent as PDGF-AA, and that PDGFR α blocking antibodies can reverse this effect, suggesting that HCMV may act through α v β 3 integrin/PDGFR α to promote glioma cell invasiveness. The HCMV encoded chemokine receptor US28 promotes cell migration toward chemokines RANTES and MCP-1 (Streblow et al., 1999), which are abundantly expressed in malignant gliomas (Desbaillets et al., 1994). In HCMV-infected neuroblastoma cells, cell invasiveness is also increased via activation of α 5 β 1 integrin and increased activity of extracellular matrix proteases (Scholz et al., 2000). As stated, HCMV infection activates the COX-2 and prostaglandin synthesis pathway. The downstream products of this pathway are also known to promote migration and invasion in gliomas and other tumor types (Tsujii et al., 1997; Tsujii et al., 1998; Zhu et al., 1998).

HCMV activation of glycolytic pathways may promote cancer

Tumor cells have high rates of glucose consumption and lactate production (and do not oxidize glucose completely), a process known as the “Warburg” effect which is considered a metabolic hallmark of aggressive tumors (Warburg, 1925). Thus, transformed cells exhibit a high rate of glutamine consumption, exceeding the requirement for protein and nucleotide synthesis (DeBerardinis et al., 2007). Glutamine metabolism has been shown to be essential for HCMV infection (Chambers et al.) and other studies demonstrate that HCMV infection induced transcriptional upregulation of enzymes involved in glycolysis and citric acid cycle, thus altering the cellular metabolism towards an environment favorable for progressing through all infection stages (Munger et al., 2006). These data suggest that HCMV infection may promote activation of glycolytic pathways associated with a highly malignant phenotype.

HCMV microRNAs and human cancer

Several microRNAs have been identified for HCMV (Pfeffer et al., 2005) as key posttranslational regulators of gene expression, functioning similarly to the eukaryotic mRNA. Viral mRNA can directly alter host cell physiology and recent data suggest that HCMV encoded micro-RNAs are involved in regulating cell proliferation (Gottwein and Cullen, 2008; Grey et al., 2007). HCMV microRNA miR-UL112 downregulates expression of MHC class I related chain B (MICB) mRNA and thus inhibits activation of natural killer cells and T cells, enabling immune evasion of the infected cells (Nelson, 2007; Stern-Ginossar et al., 2007). Another non-coding CMV RNA termed $\beta 2.7$ has been shown to bind components of the mitochondrial respiratory chain complex I and stabilize it to prevent apoptosis of infected cells (Nelson, 2007; Reeves et al., 2007). Both above mentioned effects are consistent with the hypothesis that infected cancer cells confer a survival advantage to the tumor. Functional conservation among micro RNAs from different herpes viruses whose function is to inhibit expression of the NK cell ligand MICB has been noted in spite of lack of sequence homology among these micro RNAs. This can be explained by the fact that inhibition of MICB is critical for viral immune evasion, a mechanism shared by all herpes viruses (Nachmani et al., 2009).

SUMMARY AND CONCLUSIONS

The last decade has seen an increased appreciation of the role of chronic inflammation, tumor microenvironment, cancer stem cells, tumor immunology and infectious agents in the pathobiology of cancer. We believe that sufficient evidence exists to implicate a potential role of HCMV in oncogenesis via HCMV-mediated effects of cells in the tumor microenvironment and in the tumor cells themselves. In the case of malignant glioma, several of the modified Koch's postulates of Fredericks and Relman (Fredericks and Relman, 1996) appear to be present, suggesting that HCMV may eventually be considered a tumor promoting virus in this malignancy. Whether or not HCMV is ultimately established as an oncogenic virus in malignant gliomas and/or other tumor types will still require significant work by investigators in the virology, epidemiology and molecular oncology fields. Ultimately, an increased understanding of the role of HCMV in oncogenesis may lead to unexpected shifts in our understanding of cancer biology and to the development of novel therapeutic strategies for these fatal diseases.

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