Oncomodulatory signals by regulatory proteins encoded by human cytomegalovirus: a novel role for viral infection in tumor progression

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Abstract

A high frequency of human cytomegalovirus (HCMV) genome and antigens in tumor samples of patients with different malignancies is now well documented, although the causative role for HCMV in the development of the neoplasias remains to be established. HCMV infection can modulate multiple cellular regulatory and signalling pathways in a manner similar to that of oncoproteins of small DNA tumor viruses such as human papilloma virus or adenoviruses. However, in contrast to these DNA tumor viruses, HCMV infection fails to transform susceptible normal human cells. There is now growing evidence that tumor cells with disrupted regulatory and signalling pathways enable HCMV to modulate their properties including stimulation of cell proliferation, survival, invasion, production of angiogenic factors, and immunogenic properties. In contrast to previously suggested “hit and run” transformation we suggest that persistence in tumor cells is essential for HCMV to fully express its oncomodulatory effects. These effects are observed particularly in persistent HCMV infection and are mediated mainly by activity of HCMV regulatory proteins. In persistently HCMV-infected tumor cell lines – a selection of novel, slowly growing virus variants with changes in coding sequences for virus regulatory proteins takes place. As a result, oncomodulatory effects of HCMV infection may lead to a shift to more malignant phenotype of tumor cells contributing to tumor progression.

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Keywords: Human cytomegalovirus; Oncomodulation; Tumor; DNA-virus; Apoptosis; Angiogenesis

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1. Introduction

Detection of viral DNA, mRNA and/or antigens in tumor tissues as well as seroepidemiologic evidence has implicated human cytomegalovirus (HCMV) infection in the etiology of several human malignancies (Table 1). HCMV infection was associated with Kaposi’s sarcoma [1–3], colon carcinoma [4,5], prostate adenocarcinoma [6,7], cervical carcinoma [8–10], Wilms’ tumor and neuroblastoma [11,12]. Recent investigations have revealed a higher frequency of HCMV in tumor tissues of malignancies, such as Epstein–Barr virus (EBV) negative Hodgkin’s disease [13], colon cancer [14], and malignant glioma [15], than was reported in earlier publications. This could be attributed to the limitations in technology and reagents available at the past two decades. Although these findings have renewed interest in the oncogenic potential of HCMV, a causative role for HCMV in the development of the neoplasias still remains to be established. It has been proved that HCMV infection can modulate multiple molecular pathways involved in signal transduction of cellular activation (for reviews see [16–19]). The oncomodulatory effects of HCMV on cellular metabolism are similar to those mediated by small DNA tumor viruses such as simian virus 40 (SV40), human adenovirus and human papilloma virus types 16 and 18 (HPV 16/18). In tumor cells HCMV-encoded regulatory proteins interfere with a variety of cellular signal transduction pathways leading to accelerated cell proliferation, enhanced survival, angiogenesis, cell motility and adhesion, thus enhancing the malignant behavior of tumor cells. On the other hand, HCMV-specific entities are not detectable in long-term subcultures of tumor tissues. The presence of HCMV-coded information in human tumors is difficult to interpret because HCMV can infect various organs latently in a high percentage of normal persons, and seroepidemiologic studies linking HCMV infection with malignancies have often presented conflicting results.

To study the long-term effects of HCMV infection we have established a preclinical model of persistent HCMV infection in tumor cells. In this model, HCMV-infected tumor cells provided the genetic background necessary for oncomodulatory effects of HCMV, which cannot be otherwise manifested in normal cells. Unlike HCMV-induced growth transformation of rodent cells following the hit-and-run mechanism [20,21], our studies indicate that the persistent viral infection of tumor cells is essential for HCMV to express its oncomodulatory effects [22]. Thus, HCMV could support tumor progression without having to conform to a paradigm of viral oncogenesis established by Henle’s modified Koch’s postulates [23] (Table 2). This review focuses on recent advances in our understanding of mutual interactions between HCMV and cellular functions with respect to malignant behavior of tumor cells.

Table 1
Association between HCMV infection and human malignancies

<table>
<thead>
<tr>
<th>Tumor</th>
<th>HCMV markers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoma of the cervix</td>
<td>Antibody titres higher in carcinoma patients than in control</td>
<td>[8,9]</td>
</tr>
<tr>
<td></td>
<td>HCMV DNA in tumor tissues</td>
<td>[10]</td>
</tr>
<tr>
<td>Adenocarcinoma of the prostate</td>
<td>HCMV DNA, RNA, and antigens in tumor tissues</td>
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<td></td>
<td>HCMV-specific cytotoxic lymphocytes</td>
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<td>Kaposi’s sarcoma</td>
<td>HCMV DNA in tumor tissues</td>
<td>[2]</td>
</tr>
<tr>
<td></td>
<td>HCMV RNA in tumor tissues</td>
<td>[3]</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>Antibody titres higher in sarcoma patients than in control</td>
<td>[1]</td>
</tr>
<tr>
<td>Cutaneous T-cell lymphomas (myosis fungoides; Sezary Syndrome)</td>
<td>Antibody titres higher in lymphoma patients than in control</td>
<td>[11,12]</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>HCMV DNA and antigen in tumor tissues</td>
<td>[4,5,14]</td>
</tr>
<tr>
<td>Malignant glioma</td>
<td>HCMV DNA and antigens in tumor tissues</td>
<td>[15]</td>
</tr>
<tr>
<td>Epstein–Barr virus negative Hodgkin’s disease</td>
<td>HCMV DNA and antigens in tumor cells</td>
<td>[13]</td>
</tr>
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</table>
2. Impact of DNA viruses on cell cycle and cellular DNA synthesis

The natural host cell for DNA viruses is a quiescent cell that presents an environment not conductive to viral DNA replication. The strategy of these viruses is to induce cellular mechanisms that support viral DNA synthesis. Thus, DNA viruses alter the environment of a quiescent host cell by deregulating the entry of normal cells into S-phase in order to induce the genes encoding activities essential for viral DNA replication. The transforming genes of the oncogenic small DNA viruses, such as HPV16/18, adenoviruses and SV40, provide important functions early in the replication cycle before the onset of viral replication. If the viral replication cycle fails to proceed past the expression of early gene products, the result is a persistently infected, transformed cell. As shown in Fig. 1, the oncoproteins of several DNA tumor viruses inhibit the functional activity of two key tumor suppressor genes, p53 and retinoblastoma protein (pRb), controlling progression through the G1 phase of the cell cycle (for review see [24–26]). For example, E6 protein of HPV16 and 18 interacts with p53, inducing its proteolytic inactivation, while adenovirus 5 (AD5) E1-B 55 kDa protein and SV40 large T antigen bind to p53, forming stable inactive complexes. The binding of E7 of HPV 16 and 18, E1A of AD5, or large T antigen of SV40 to pRb may lead to its inactivation. In addition to the interaction with tumor suppressor genes, viral oncoproteins can influence cell-cycle progression by effecting the expression and activity of positive regulators, i.e., complexes of cyclins and their
catalytic partners, the cyclin-dependent kinases (Cdks), as well as negative regulators of the cell cycle, including members of both the KIP family (including p21, p27 and p57) and INK4 family (including p15, p16, p18 and p19) (Fig. 1).

2.1. Impact of HCMV on cell cycle

In recent years, it has become apparent that HCMV-coded proteins can deregulate the cell cycle in a manner similar to small DNA tumor viruses. Accumulating evidence suggests that different viral proteins, including two virion-tegument proteins (pUL69 and pp71), immediate early (IE) proteins, and proteins encoded by the morphological transforming region II (mtrII), interact with key cell-cycle regulatory proteins (Fig. 2). The IE1-72 and IE2-86 proteins of HCMV can bind to members of the Rb family, the so-called pocket proteins p107, p130, and pRb; however, they do not bind to pocket domains, which is most likely due to the absence of the consensus pocket domain-binding motif, LXCXE [19,27]. Oncoproteins encoded by small DNA tumor viruses do bind to the pocket domain. The IE1-72 protein interacts with the p107 protein [28], while the IE2-86 protein binds to pRb [29,30]. Analogous to the oncproteins of small DNA tumor viruses, IE1-72 and IE2-86 expression can alleviate the repression of transcriptional activity of E2F family members mediated by p107 and pRb. In addition, pp71 phosphoprotein expressed from UL82 ORF, binds to all three Rb family members through an LXCXD motif in vitro and induces DNA synthesis in quiescent (G0) cells [31–33]. The function of pp71 is to direct proteasome-dependent, ubiquitin-independent degradation of the Rb family proteins in the absence of other viral proteins [32,34]. IE2-86, but not IE1-72, has been shown to interact with p53 in vitro and in vivo, and this interaction results in the down-regulation of p53 transactivation function [35,36]. Even in the presence of functional p53, IE2-86 can block downstream of p53 cell-cycle arrest pathways by degradation of p21[CIPI]/WAF1 [37]. HCMV “morphological transforming region II” (mtrII) encodes the ORF 79 (UL111A) protein, which has been reported to bind p53 and disrupt its cellular transcription activity [38]. So far, mtrII is the only HCMV gene yet reported to be retained after induction of transformation in a susceptible murine NIH 3T3 cell line, which was tumorigenic after inoculation into immunodeficient nude mice [39].

HCMV-mediated inactivation, or down-regulation of tumor suppressor proteins controlling the cell cycle supports the findings that HCMV has positive effects on promoters of cell proliferation. Among many factors that are up-regulated during HCMV infection, the proto-oncogenes c-myc, c-fos, and c-jun are rapidly up-regulated.

Fig. 2. A summary of the effects of human cytomegalovirus (HCMV) on key regulators of cell cycle and apoptosis. Infection with HCMV leads to specific alterations in the factors that regulate progression through the cell cycle. Even though the HCMV tegument pUL69 protein may prevent S-phase progression, several HCMV proteins including IE1-72, IE2-86 and tegument protein pp71 interact and inactivate proteins of pRb family, thus resulting in activation of E2F transcription factor and subsequent stimulation of expression of several S-phase genes. HCMV induced E2F may activate transcription of p19ARF (p14ARF in humans), which is important positive regulator of p53 tumor suppressor gene via inactivation of Mdm2 (Hdm2 in humans), negative regulator of p53. In this way, HCMV proteins may activate p53 and promote its function in induction of apoptosis or cell cycle arrest. However, several HCMV proteins may counteract these effects. HCMV IE2-86 and mtrII interact with p53 leading to down-regulation of p53 transactivation function. Moreover, HCMV IE2-86 inactivates cyclin/Cdk inhibitor p21[CIPI] by induction of its degradation. p53 dependent apoptosis may also be prevented by effects of HCMV on its downstream molecules. HCMV up-regulates Bcl-2 (at least in some tumor cells) by unknown mechanism and HCMV IE protein pUL37x1/vMIA inhibits apoptosis due to its Bcl-2-like activity. Additionally, pUL36/vICA inhibits apoptosis by blocking efficient cleavage of procaspase-8.
activated in infected cells [40]. Additionally, viral infection activates several cellular S-phase genes, including DNA polymerase alpha, dihydrofolate reductase (DHFR), and thymidine kinase (TK). HCMV also stimulates expression of cyclin E and Cdk2 proteins which commit the cell to DNA replication at the end of G1 [41–43]. The importance of cyclin E for HCMV infection was shown by the demonstration that inhibition of its associated kinase, Cdk2, blocks HCMV replication [44]. However, the expression of cyclin A, the S-phase cyclin, is delayed as a result of HCMV-infection [45]. This is in sharp contrast to infection with small DNA viruses that stimulate both cyclin A and Cdk2. HCMV-induced cyclin E/Cdk2 activity is probably responsible for the increased levels of hyperphosphorylated pRb in infected cells [45]. Since IE2-86 does not bind the phosphorylated form of pRb [29], it is likely that the HCMV-induced cyclin E/Cdk2 activity inhibits the interaction between pRb and IE2-86. However, the findings that most of S-phase genes induced by HCMV infection are E2F targets [46,47] demonstrate that the virus can inactivate the ability of pRb family proteins to repress E2F-dependent transcription.

One may speculate that the interactions between HCMV and cellular tumor suppressor proteins as well as stimulation of S-phase genes result in the inactivation of required checkpoints in the cell cycle, giving rise to uncontrolled cellular DNA replication and transformation. However, in normal permissive fibroblasts just the opposite is true. HCMV arrests cells in both G1/S and G2/M transition points of the cell cycle [45,48–50]. Consistent with the G1/S growth arrest phenotype described in HCMV-infected fibroblasts, another study demonstrated that IE2-86 expression led to a cell cycle arrest at the G1 phase [51,52]. This particular effect of HCMV or IE2-86 protein resembles premature senescence [52], which represents one of the important mechanisms to prevent uncontrolled proliferation and a risk of oncogenic transformation [53]. In HCMV-infected fibroblasts with the premature senescence phenotype and the accumulation of large amounts of the p53 and pRb (mainly in its hypophosphorylated form), a strong induction of the Cdk inhibitor p16INK4a, a direct effect of the senescence phenotype in fibroblasts, and a decrease of p21CIP1/WAF were found [52]. The pUL69-mediated G1 arrest is still not well characterized; however, it is believed to be important for the full expression of cell cycle arrest by HCMV [54].

2.2. Impact of HCMV on cellular DNA synthesis

HCMV infection of human fibroblasts has been shown to prevent cellular DNA replication through inhibition of the reactivation of origins of replication, i.e., replication licensing (Fig. 3) [55,56]. The process of replication licensing represents a control mechanism that allows DNA

![Fig. 3. Model for the inhibition of replication licensing in HCMV infected fibroblasts. In non-infected fibroblasts formation of the pre-RC occurs in G1 by sequential loading of Cdc6, Cdt1 and MCM proteins on an origin of DNA replication. Activation of pre-RC or origin firing occurs in S phase under the influence of S-phase Cdk. Formation of pre-RC is prevented during the remainder of the S, G2, and M phases mainly by the presence of the replication inhibitor geminin. In HCMV infected cells, accumulation of geminin inhibits the loading of MCM proteins onto the cellular origins of replication possibly through interaction of geminin with Cdt1. Moreover, HCMV IE2-86 may inhibit licensing at cellular origins through binding with MCM3 and MCM7 to provide at the same time the viral origins of replication with helicase activity of MCM.](https://academic.oup.com/femsre/article-abstract/28/1/59/635661)
generalized and viremia is detectable from a few weeks to a few months. Consequently, during the viremic phase of HCMV infection a low level of lytic replication has to be balanced by the replication of non-infected cells. Since HCMV infection can lead to (over)expression of growth factors in host cells, it is probable that the growth and differentiation of neighboring (non-infected) cells is indirectly influenced. Human cell lines persistently and productively infected with HCMV provide a more appropriate model than acutely infected cultures for studying the role of HCMV in oncomodulation. It has been demonstrated that persistent infection of tumor cells, including osteogenic sarcoma [61], glioblastoma [62], and neuroblastoma [16], results in the development of mutated HCMV variants, which grow slowly and yield lower amounts of progeny virus compared to wild-type virus strains originally used for infection. These HCMV variants were found to have DNA deletions in their genome [61] and synthesized IE proteins different in size from those of the wild-type virus strain [62]. Similarly, stable expression of HCMV IE2-86 kDa protein in retrovirus-transduced fibroblasts did not abrogate the G1 checkpoint, despite its binding to p53 [63]. These properties of IE2-86 in stable transduced cells were shown to be due to a mutation within a critical carboxyl-terminal domain of the protein, thus making it unable to halt cell cycle progression [51,64]. The mutations in the IE2-86 gene, accounting for changes in its transcriptional activity, result from alterations in its post-translational modifications including phosphorylation and sumoylation [65]. Infection of fibroblasts with HCMV containing a deletion in the UL69 gene failed to induce a block in the G1/S phase of the cell cycle. However, this was reversed by incorporation of the ppUL69 protein into mutant virions [54]. As with HCMV variants from persistently infected tumor cell lines, the UL69-mutated virus exhibited slower growth and delayed virus production. Possibly the virus variants, with regulatory proteins that have lost their ability to induce cell cycle arrest, develop in persistently infected tumor tissues of patients.

Moreover, many regulators of the cell cycle are inactivated in tumor cells. This may be important for the effects of HCMV infection on the cell cycle. It has been shown that in diploid fibroblasts expressing wild-type p53, HCMV IE1-72 protein cannot drive cells out of quiescence, while, in cells deficient for p53, IE1-72 expression can induce S phase and delay cell cycle exit [50]. HCMV IE2-86 protein induced a G1/S block in human cells with wild-type pRb [51], but not in the human osteosarcoma cell line Saos-2, deficient for pRb [30]. In addition, in human U3T3MG glioblastoma cells with mutations in the p16INK4a and p53 pathways, HCMV infection was not able to induce premature senescence [52]. It is possible that the effects of HCMV on replication licensing depend also on the
status of pRb and p53. The overexpression of geminin in U2OS (p53+/Rb+) cells induced early S-phase arrest with high cyclin E and undetectable cyclin A levels [66], which resembles the cell cycle arrest induced by HCMV in fibroblasts [60]. In contrast, in Saos-2 (p53−/Rb−) cells geminin failed to induce early S-phase arrest and inhibit DNA replication [66]. Moreover, geminin expression was found in several malignancies and the number of geminin-positive cells was directly proportional to the proliferation index [67]. One can postulate that the inability of geminin, overexpressed in HCMV-infected cancer cells, to induce cell cycle arrest is attributed to deregulation of the p53 and pRb pathways.

Given a requirement for persistent productive HCMV infection in tumor tissues, lytic viral replication has to be balanced by the replication of non-infected cells [16]. Therefore, mechanisms that restrict virus replication to avoid excessive cell killing must exist. As mentioned above, HCMV variants may develop in persistently infected cells that have a tendency to grow slowly. Tumor cells may escape HCMV-induced death through the ability to express only a subset of viral genes as a consequence of the restriction of viral replication by specific cellular factors. Human HOG oligodendroglioma cells are relatively non-permissive to HCMV under normal growth conditions, in the sense that HCMV IE mRNA synthesis is delayed and only small amounts of IE proteins are expressed [68]. However, upon differentiation, HOG cells can express high levels of IE antigens and also synthesize infectious virions. Similarly, human A172 and T98G glioblastoma cells, also exerted restricted IE expression upon HCMV infection [69,70]. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) may also modulate HCMV replication [71,72]. These enzymes are closely linked with the regulation of the cell cycle and DNA replication [73,74]. Together, HATs and HDACs determine the acetylation status of histones, thus affecting the regulation of gene expression. Tumor cells frequently exert disrupted activity of HATs and HDACs [75]. Human teratocarcinoma cells containing the class I HDAC (HDAC3) are unable to replicate HCMV. Inhibition of HDAC3 by specific inhibitors or as a result of cell differentiation, enhanced repression of the major IE promoter and supported virus replication [71]. HCMV infection of human fibroblasts during S phase resulted in a blockade to IE gene expression. This blockade was overcome by inhibition of proteasome function suggesting that a short-lived protein in S-phase cells is required for IE gene expression [76]. The restricted expression of IE genes in replicating cells may be important not only to limit HCMV replication but may also attenuate inhibitory effects of infection on cell cycle progression. In fact, it has been demonstrated that S-phase infected cells maintain the ability to divide [76,77]. These findings suggest that rapidly dividing cells provide an environment not conducive to viral replication and that there is a switch to productive infection when the cells no longer divide. Additionally, in HCMV-infected cells the host and virus factors may cooperate to induce IFN-β, in which state the host cell survives and the viral genome persists, but cannot produce new virions [78]. This state of viral persistence is maintained due to signals via TNF receptor1 (TNFR1), or the lymphotoxin β receptor, which overrides IFN-β inhibition induced by HCMV in permissive cells [79]. It is conceivable that TNF-α, which is frequently produced during tumor progression, contributes to HCMV persistence. Thus, one can speculate that different mechanisms are involved in the persistence of HCMV in tumor cells without induction of cell cycle arrest and cell lysis (Fig. 4).

3. Oncomodulation and apoptosis

Apoptosis can serve as a defense mechanism for the host organism to combat viral infection. The apoptotic response involves a cascade of intracellular signals initiated in response to a wide variety of stimuli, including viral infection. Apoptosis can manifest itself in two non-exclusive manners, i.e., the death receptor (extrinsic) pathway and the mitochondrial death (intrinsic) pathway.

It has been demonstrated that small DNA tumor viruses can modulate both pathways, resulting in inhibition of apoptosis (for review see [80]) (Fig. 5). The interaction of oncoproteins of small DNA tumor viruses with key tumor suppressor proteins pRb and p53 may prevent their function in either growth arrest (as outlined above) or apoptosis, depending on the context of cellular environment. In addition to such effects, oncoproteins of small DNA viruses may suppress apoptosis directly. For example, the adenovirus inhibits apoptosis via E1A-19 kDa oncoprotein, which is a Bcl-2 homolog that forms heterodimers with proapoptotic proteins such as Bax [81]. The E6 oncoprotein of HPV 16 stimulates not only proteolytic inactivation of p53 but also of other proapoptotic proteins including Bak, Bax and c-Myc [25]. Several adenovirus proteins encoded by the E3 region (E3 – 6.7 kDa, E3 – 10.4 kDa and E3 – 14.5 kDa) were shown to prevent apoptosis induced by death receptor pathways through down-regulation of death-inducing receptors [80]. Moreover, E6 of HPV 16 is able to prevent the interaction of the intracellular death domain of the “death receptors” such as CD95/FAS with the cytosolic adaptor Fas-associated death domain (FADD), and thus interrupting the extrinsic apoptosis pathway [25]. HPV 16/18 E7 and SV40 middle T
oncoproteins sustain activity of cellular kinase Akt probably through the upstream phosphatidylinositol 3-kinase (PI3-K) \[82,83\]. This may be important for inhibition of apoptosis, as Akt is a general mediator of cell survival. Indeed, analysis of a polyoma virus mutant defective in binding and activating PI3-K provided evidence of a role of PI3-K/Akt signalling in blocking apoptosis by polyoma viruses \[83\].

Fig. 4. Model of persistent HCMV infection in tumor cells. Infection of diploid fibroblasts permissive for HCMV is characterized by establishment of conditions conductive to viral replication. High levels of IE1/IE2 proteins are synthesized contributing to stimulation of S-phase genes but host cell is actually blocked in S-phase progression. In this state vigorous viral replication is promoted, resulting in high production of progeny viruses and cell lysis. The establishment of persistent infection in tumor tissues is based on the balance between infected and non-infected tumor cells, i.e., between release of the virus and growth of non-infected cells. In this state low production of slowly growing virus variants occurs. These variants are not able to induce cell-cycle arrest and efficiently replicate probably due to mutations in viral genes coding for regulatory proteins. A selection pressure for development of such variants results from cellular context of tumor cells. Cellular factors produced during S-phase suppress IE1/IE2 expression, which together with inactivation of cellular regulatory and signaling pathways may contribute in escaping from the cell-cycle arrest and premature senescence. Moreover, environmental influences such as TNFR1 signalling (not shown) may contribute to HCMV persistence in tumor cells.

Fig. 5. Possible mechanism of immune escape of HCMV-infected cells due to up-regulation of FasL. (A) Non-infected tumor cells expressing the Fas receptor and low FasL are killed through Fas/FasL-mediated apoptosis by cytotoxic CD8+ cells recognizing the tumor antigen or NK cells. (B) Up-regulation of FasL on infected tumor cells induced by HCMV IE proteins may result in Fas/FasL interaction on Fas positive immune cells and result in their elimination.
3.1. Effects of HCMV proteins on apoptosis

By analogy with small DNA tumor viruses, HCMV may prevent apoptosis by different mechanisms. HCMV IE2-86 was shown to bind to p53 and suppress its transactivating function, which is important for induction of apoptosis depending on the specific cellular context [35,36]. IE2-86 inhibits doxorubicin-induced apoptosis in smooth muscle cells, indicating that IE2-86 can suppress p53-mediated apoptosis following DNA damage [84]. Furthermore, ts13 cells with a temperature-sensitive mutation in TAFII250, do not undergo p53-dependent apoptosis when IE2-86 is expressed and the cells are grown at the non-permissive temperature [85]. IE2-86 also blocked p53-independent apoptotic pathways such as the TNF-mediated death receptor-signalling pathway [86], but, on the other hand, failed to protect cells from apoptosis following UV irradiation [85].

Direct anti-apoptotic activity of HCMV proteins was ascribed mainly to some distinct IE transcripts encoded by the HCMV UL36–UL38 genes. In addition to 11 gene products expressed from these loci essential for viral DNA replication [87], the two IE proteins encoded at these loci have been reported to exhibit anti-apoptotic activity; namely, the product of UL36 ORF, a viral inhibitor of caspase activation (vICA) [88], and the UL37 ORF gene product, pUL37 exon 1 (pUL37x1), a viral mitochondrial inhibitor of apoptosis (vMIA) [89]. vMIA is a type-1 heavily glycosylated transmembrane protein localized primarily in mitochondria following HCMV infection, and inhibiting mitochondrial megapore activation in a manner similar to members of the anti-apoptotic Bcl family [89]. It has been shown that HeLa cells expressing pUL37x1 suppressed intrinsic apoptosis triggered by doxorubicin [89]. In mitochondria, vMIA forms a complex with an adenine nucleotide translocator, believed to be a component of the mitochondrial transition pore complex [90]. Thus, it suppresses apoptosis by blocking permeabilization of the mitochondrial outer membrane. This is the only UL37 function that is essential for viral replication in cultured cells, and UL37 is the leading candidate responsible for preventing virus-induced, intrinsic apoptosis. The biological functions of two longer UL37 splice-variant proteins, gpUL37 and gpUL37M, are not yet known, and may be unrelated to their anti-apoptotic activity [89]. Both gpUL37 and gpUL37M are dispensable for virus replication in cultured cells as long as vMIA/pUL37x1 is present [89,91]. Despite the similarity of behavior to anti-apoptotic members of the Bcl-2 family, vMIA does not share any significant amino acid sequence homology with Bcl-2, and, unlike Bcl-2 or Bcl-xL, it does not bind proapoptotic Bcl family members or voltage-dependent anion channels. These structural and functional differences between vMIA and Bcl-2 suggest that vMIA represents a separate class of cell death suppressors. vMIA is a broadly acting inhibitor that can suppress apoptosis not only induced by stimuli of the mitochondrial pathway, but also by stimuli of the death receptor pathway.

HCMV vICA/pUL36 protein protects cells from extrinsic apoptosis induced via the death receptors including TNFR1, Fas/CD95 or Trail [88] (Fig. 5). The fact that UL36 is mutated in many laboratory-cultured HCMV strains indicates that it plays no role during viral replication in culture. The expression of UL36 blocks the processing of procaspase-8 (FLICE) by direct physical interaction with the caspase pro-domain [88]. In this respect pUL36 exhibits behavior similar to both viral and cellular FLICE-ligand inhibitory proteins (FLIPs), even though the HCMV protein shares no primary amino acid sequence similarity and has no candidate death effector domains in common with FLIPs. The viral and cellular proteins that are linked with apoptosis at this stage either interfere with the zymogen itself (vFLIPs and cFLIPs) or inhibit protease activity (serpins) [80]. vICA is analogous to a FLIP. Thus, UL36 plays no apparent role in counteracting intrinsic pro-apoptotic signals during viral infection but is likely encoded by the virus to modulate cytokine- and/or death receptor-mediated apoptosis.

An interesting mechanism by which HCMV interacts with extrinsic apoptotic pathways represents its stimulatory effect on expression of death receptor ligands (Fig. 6). HCMV was shown to up-regulate membrane-bound FasL in human retinal pigment epithelial cells via IE2-86 protein [92,93], while in fibroblasts HCMV infection induced TRAIL [94]. The up-regulation of death receptor ligands may represent a viral immune evasion tactic through the killing of infiltrating host Fas- and TRAILR1/TRAILR2-positive immune cells [80,92]. The effects of HCMV on expression of death receptor ligands in tumor cells have not yet been studied. However, since FasL expression on tumor cells of different malignancies may correlate with tumor progression, the up-regulation of death receptor ligands may represent an important oncomodulatory mechanism of HCMV. Additionally, HCMV contains a TNFR ortholog encoded by the UL144 orf [95]. Neutralization of TNF by soluble viral decoy receptors was one of the first-described immune evasion tactics, as shown by the secreted ortholog expressed by tumorogenic Shope fibroma virus (rabbit poxvirus) [96]. However, the functional significance of HCMV TNFR ortholog remains obscure [80].

3.2. Induction of Akt (PKB) by HCMV

The cellular kinase c-Akt (also known as protein kinase B, PKB) is a cellular homologue of the oncoprotein of AKT8 retrovirus (for reviews see [97,98]). The cellular
c-Akt is activated by PI3-K in response to many factors, including insulin, and insulin-like growth factor (IGF) [97]. The c-Akt signalling pathway was originally studied as a regulator of glucose uptake and metabolism. Akt is an essential gene in the maintenance of normal glucose homeostasis. Recently, Akt was also described as a general mediator of cell survival. Akt functions in an anti-apoptotic pathway: dominant negative alleles of Akt block IGF1-mediated cell survival, and constitutively active Akt rescues PTEN-mediated apoptosis [98]. The mechanism by which Akt protects cells from death is most likely multifactorial, since Akt has the ability to phosphorylate several components of the cell death machinery. For example, Bad is a pro-apoptotic member of the Bcl-2 family of proteins that promotes cell death by forming a non-functional heterodimer with the survival factor Bcl-xL. Phosphorylation of Bad by Akt prevents this interaction, restoring Bcl-xL’s anti-apoptotic function [99]. Similarly, Akt inhibits the catalytic activity of a pro-death protease, caspase-9 by phosphorylation [100]. Finally, Akt phosphorylates FKHR, a member of the Forkhead family of transcription factors, and prevents its nuclear translocation and activation of FKHR gene targets, which include several proapoptotic proteins such as BIM and Fas ligand [101]. Moreover, Akt can also influence cell survival by indirect effects on two central regulators of cell death, nuclear factor κB (NF-κB) and p53 [98].

Some herpes viruses such as EBV and HCMV can activate Akt. Recently, it has been shown that HCMV infection can activate the PI3-K pathway [102], suggesting that viral structural components and/or viral proteins produced in the infected cells may affect the activity of Akt. It has been demonstrated that HCMV-induced activation of Akt alone can inhibit apoptosis in a temperature-sensitive ts13 cell line grown at the non-permissive temperature [103]. These effects were induced by IE1-72 and IE2-86 proteins via activation of PI3-K. The HCMV-induced Akt activation may be important for coordination of alterations in key cellular processes such as glucose uptake and metabolism, the cell cycle and apoptosis. In normal cells Akt activation is proba-
bly required to provide a metabolic advantage for HCMV replication at late phases of the infectious cycle. However, for restriction of HCMV replication in tumor cells, HCMV-induced Akt activation may be important for promoting malignity, i.e., the Akt-induced metabolic changes may be of benefit for tumor cell growth and inhibition of apoptosis [98]. For example, activation of Akt was shown to play an important role during the progression of colorectal carcinoma [104], which is frequently associated with HCMV infection. In addition to helping promote cell growth and rescuing tumor cells from apoptosis, Akt phosphorylation was correlated with some clinico-pathological parameters of the malignancies, including depth of invasion, infiltration to venous vessels, lymph node metastasis, and stage of disease [104].

3.3. Induction of ΔNp73 by HCMV

A close homologue of p53, termed p73, expressed in different isoforms depending upon the mode of splicing of mRNA, may be also involved in apoptotic inhibition by HCMV infection. The p73 isoforms induce p53-responsive promoters by their transactivating domain (TA), and they cause growth arrest or trigger apoptosis similar to p53 [105,106]. p53 seems to require p63 and p73 to induce apoptosis, indicating a very tight relationship between the three members of the same family [107]. Despite the similar set of target genes, a number of biological differences were found between p53, p73 and the third family member, p63/p51. Oncoproteins of small DNA tumor viruses, including the adenovirus E1B 55 kDa, HPV 16 E6 and SV40 large T antigen, inactive p53, but not p73 [106,108,109]. Unlike p53, the p73 and p63 genes do not represent “bona fide” tumor suppressor genes, and tumors rarely contain mutations in p73 and p63. The p73 gene was found to contain a second transcriptional start site, giving rise to the expression of ΔNp73, p73 proteins that lack the N-terminal transactivation (TA) domain. These ΔNp73 proteins have various carboxyterminal ends, due to differential splicing at the 3′ portion of the mRNA, yielding isoforms analogous to the TA p73 proteins. It has been suggested that ΔNp73 overcomes p53-induced growth arrest or apoptosis, at least when mdm2 and the related mdmx protein are not expressed in amounts sufficient to do so [105,106]. Recent observations showed that HCMV infection induced robust accumulation of ΔNp73isoforms through virus-induced protein stabilization [110]. Anti-apoptotic effects of HCMV were observed in both p53-negative tumor glioblastoma U373MG cells and neuroblastoma IMR-32 cells expressing functional p53. HCMV-induced ΔNp73exoerts a dominant negative effect on both p73- and p53-dependent apoptosis. Although specific viral components inducing elevated ΔNp73 levels have not yet been identified, the levels of ΔNp73 were up-regulated as early as 4 h after infection, suggesting that viral replication is not essential for the observed effects. Interestingly, the expression of the ΔNp73 isoform, but not TA p73 correlated with both poor overall and progression-free survival in neuroblastoma patients [111], suggesting that HCMV may promote neuroblastoma due to induction of ΔNp73.

3.4. Induction of Bcl-2 family members by HCMV

An important mechanism of resistance to apoptosis in HCMV-infected tumor cells may be the induced expression of anti-apoptotic proteins of the Bcl-2 family. Members of this family, including numerous anti-apoptotic (Bcl-2, Bcl-xL, A1 and McI1) and pro-apoptotic (Bax and BH3-only families) proteins, govern a commitment to apoptosis in response to diverse physiological stimuli and cytotoxic agents [112,113]. Bcl-2 and its closest pro-survival relatives Bcl-xL and Bcl-w associate with the mitochondrial outer membrane and the endoplasmatic reticulum/nuclear membrane and maintain their integrity [113]. Initiation of apoptosis requires pro-apoptotic family members such as Bax and Bak that closely resemble Bcl-2, especially in Bcl-2 homology (BH) domains, including BH1, BH2 and BH3. Either Bax or Bak is required for apoptosis, but how they are activated or countermanded by Bcl-2 remains uncertain. During apoptosis Bax and Bak oligomerize in the mitochondrial outer membrane and probably breach its integrity, freeing proapoptotic proteins such as cytochrome c, which allows activation of caspase 9 [114,115]. The pro-survival Bcl-2 proteins or their relatives can prevent cytochrome c release and hence caspase 9 activation [113]. The other pro-apoptotic proteins such as Bid, Noxa and Puma have only a short BH3 motif (hence their name). These BH3-only proteins (with the possible exception of Bid) act as damage sensors and direct antagonists of the pro-survival proteins of Bcl-2 family [113]. The BH3-only proteins cannot cause cell death in the absence of Bax and Bak, and hence must function upstream in the same pathway. As shown recently, HCMV infection inhibited apoptosis induced by growth factor withdrawal in primary endothelial cell cultures [116]. The cellular levels of Bcl-xL were elevated in the HCMV-infected cultures, thereby suggesting that HCMV infection might upregulate expression of this anti-apoptotic protein. In addition, two independent investigators reported increased Bcl-2 expression in cultures of neuroblastoma [22] and colon carcinoma cells [14] infected with HCMV. We observed increased expression of Bcl-2 protein in persistently infected neuroblastoma cells, which are significantly less sensitive to the cytotoxic agents cisplatinum and etoposide than parental (non-infected)
cells. The inhibition of virus production through treatment of HCMV-infected neuroblastoma cells with the antiviral agent ganciclovir, restored the sensitivity to chemotherapy, lowered Bcl-2 expression, and facilitated sensitivity of apoptosis to the level of parental cell line [22]. In cultures of colon cancer cells (Caco-2), HCMV infection resulted in the induction of Bcl-2 protein. The increased Bcl-2 expression was observed in Caco-2 cells expressing HCMV IE1-72 protein but not in uninfected adjacent cells. All together these findings suggest that HCMV infection induces alteration of apoptosis and drug resistance in some tumor cells at least in part due to increased levels of Bcl-2 protein.

4. Viral effects on angiogenesis

The growth and metastasis of the majority of tumors depend on the formation of new blood vessels [117,118]. The oncogene-driven production of angiogenic factors by tumor cells promotes incorporation of vascular and lymphatic endothelial cells into the tumor vasculature by co-option, migration or proliferation of pre-existing neighboring vessels (sprouting) [119,120]. The pro-angiogenic proteins include vascular endothelial factor (VEGF), basic fibroblast growth factor (bFGF), FGF-binding protein (FGF-BP), CXC chemokines including CXCL8 (interleukin-8; IL-8) and CXCL1 (growth-related oncogene; Gro-α), placenta-like growth factor (PLGF), transforming growth factor-β (TGF-β), platelet-derived endothelial growth factor (PD-EGF), pleiotrophin and others [119,121]. Tumor-associated hypoxic conditions activate hypoxia-inducible factor-1α (HIF-1α), which promotes up-regulation of several factors [122]. Fibroblasts in or near the tumor bed begin to produce pro-angiogenic factors and tumors also recruit progenitor endothelial cells from bone marrow to build new blood vessels [123]. The promotion of angiogenesis also involves down-regulation of angiogenic suppressor proteins, such as thrombospondin 1 and thrombospondin 2 (TSP 1 and TSP 2) [119]. It has been demonstrated that small DNA tumor viruses may disturb angiogenic balance by increasing production of numerous angiogenic activators and decreasing production of angiogenic inhibitors [124,125]. Similar to cellular oncoproteins the viral oncoproteins drive production of angiogenic factors. HPV-16 E6 stimulates VEGF and FGF-BP expression by activating their gene promoter [126,127], whereas SV40 LT stimulates VEGF by inactivating of p53 [128]. The inactivation of p53 by oncoproteins of small DNA tumor viruses may have general significance for stimulation of angiogenesis since wild-type p53 has been shown to suppress tumor angiogenesis by up-regulating TSP1, inducing degradation of HIF-1α, suppressing transcription of VEGF and down-regulating FGF-BP [118,129]. Moreover, tumor cells with inactivated p53 exert decreased vascular dependence as demonstrated by maintained tumor growth and diminished rate of apoptosis under hypoxic conditions as well as resistance to inhibitors of angiogenesis [130]. Therefore, it is possible that infection with oncogenic DNA viruses improves tumor growth in hypoxic conditions due to inactivation of p53.

4.1. Effects of HCMV proteins on angiogenesis

The effects of HCMV-encoded proteins on angiogenesis have not yet been extensively studied. However, it has been shown that vCXCL1 chemokine encoded by one (UL146) of the two adjacent genes (UL146 and UL147) present in the HCMV genome [131] may play a role in angiogenesis. HCMV–CXCL1 as well as its cellular homologues CXCL1 (Gro-α) and CXCL8 (IL-8) exhibit a common repetitive amino acid sequence Glu-Leu-Arg (ELR), which is important for the functional activity of CXC chemokines. HCMV–CXCL1 is a very potent chemokine that induces chemotaxis of human neutrophils and, like cellular CXCL8 and CXCL1, induces degranulation and selective effector functions. This chemokine (similar to cellular CXCL1) binds specifically to CXCR2, one of the two human IL-8 receptors. It does not bind to any other chemokine receptors including CXCR1, the other IL-8 receptor. Human microvascular endothelial cells express the CXCR2 receptor, which plays a functional role in angiogenesis, and is induced by ELR+CXCL chemokines but not CX chemokines that lack the ELR motif [132]. CXCL1 and CXCL8 were shown to induce angiogenesis and progression of different malignancies, including those frequently infected with HCMV [133–135]. Therefore, it is probable that secretion of HCMV–CXCL1 ELR+CX chemokine from infected cells influences angiogenesis in a similar manner to its cellular homologues (Fig. 7).

4.2. Induction of cellular angiogenic molecules by HCMV

A possible involvement of HCMV infection in angiogenesis was suggested by the findings that supernatant from HCMV-infected cells stimulated proliferation of endothelial cells [136]. Basic fibroblast growth factor (bFGF) was detected as major angiogenic factor in the supernatant of the infected cells and production of bFGF was dependent on HCMV IE expression [137]. HCMV could influence tumor angiogenesis due to numerous, positive and negative, regulators of angiogenesis (Fig. 7). In addition to infection of tumor cells, HCMV could infect stromal and/or peritumoral non-transformed cells such as fibroblasts, and inflammatory cells such as macrophages and thus contribute to tumor angiogenesis. In normal cell types, including fibroblasts, smooth muscle cells, endothelial cells and macrophages,
HCMV infection was shown to up-regulate a variety of cytokines, including TGF-β, TNF-α, IL-1, IL-6, IL-8, Gro-α or prostaglandin E2 (PGE2), due to stimulation of cyclooxygenase-2 (COX-2) expression [138–145]. HCMV-induced stimulation of most of these factors relies on the activity of IE proteins and/or is regulated by virus-induced cellular transcription factors, such as NF-κB, SP-1 or AP-1 [18]. There are relatively few data that shed light on the effects of HCMV infection on production of angiogenic factors in tumor cells. Stimulation of TGF-β in HCMV-infected astrocytoma U373MG cells has been reported to be associated with the activity of IE1 and IE2 proteins [139,146]. IL-8 was induced by HCMV in the same tumor cell line by IE1-72 protein [147]. In the colon cancer cell line Caco-2, HCMV infection resulted in specific induction of COX-2 protein [14]. We have demonstrated that HCMV infection impairs the production of TSP-1 and TSP-2 in fibroblasts, retinal glial cells and glioblastoma cell lines [148,149]. The effects on TSP-1 and TSP-2 are attributed to HCMV-IE activity without the involvement of p53. Although wild-type p53 is an important transcriptional activator of TSP-1, we have shown that HCMV can exert these effects in tumor cells with mutated p53. In experimental models using nude mice, both TSP-1 and TSP-2 expression was sufficient to suppress tumor growth proportionally to the extent of neovascularization [150]. The lack of TSP-2 gene expression was significantly associated with higher histological grade and increased vessel-count density in patients with glioblastoma [151]. Expression of TSP-1 inversely correlated with tumor vascularity and hematogenous metastasis in colon cancer [152]. These findings suggest that the angiogenic response due to suppression of TSP-1 and/or TSP-2 gene expression may be an important mechanism by which HCMV infection promotes angiogenesis and tumor growth.

Another route of HCMV-induced angiogenesis may result from the ability of virus to up-regulate expression of CD40 on infected endothelial cells [153]. CD40 is a cell surface receptor that belongs to the TNF receptor family and was first identified and functionally characterized on B lymphocytes [154]. However, the distribution of CD40 appeared to be much broader than originally thought and includes expression on monocytes, dendritic cells, epithelial cells and endothelial cells [155,156]. The ligand for CD40, CD154 (CD40L) is a membrane-bound member of the TNF receptor family, which is dominantly expressed by activated CD4⁺ cells. The critical role played by this ligand–receptor pair is underscored by the fact that its blockade inhibits humoral immunity and many aspects of cellular immunity [155,157]. Increasing evidence supports the importance of CD40–CD40L interactions among endothelial cells, activated T cells and circulating monocytes in different disorders, including atherosclerosis [158] and tumor angiogenesis [159,160]. It has been demonstrated that ligation of CD40 induces the expression of VEGF by
endothelial cells, monocytes and synovial fibroblasts and promotes angiogenesis in vivo [161,162]. HCMV infection of endothelial cells was shown to increase CD40 levels on the surface of infected endothelial cells between 8 and 72 h post-infection, via de novo CD40 protein synthesis [153]. Immunocytochemical analysis of HCMV-infected tissues in vivo confirmed elevated levels of CD40 on infected endothelial cells [153]. Thus, it is important to consider to what extent HCMV infection of endothelial or other cell types may contribute to VEGF production and angiogenesis via CD40–CD40L interaction in infected tumor tissues.

5. Conclusions

HCMV proteins and DNA were detected with high frequency in tumor tissues but not in adjacent normal tissues of patients with different malignancies. The ability of HCMV to preferentially infect tumor tissues suggests a unique character of mutual interactions between the metabolism of tumor cells and HCMV. There is growing evidence that HCMV infection modulates properties of tumor cells such as growth, apoptosis, production of angiogenic factors, cell invasion and immunogenity. These oncomodulatory effects are mediated mainly by the activity of HCMV regulatory proteins and rely on the persistence of viral infection in malignant cells. In vitro persistent HCMV infection of tumor cell lines may lead to a selection of novel virus variants characterized by slow growth and changes in coding sequences for virus regulatory proteins. It is of special interest to characterize regulatory proteins of HCMV strains infecting tumor tissues and to study their interactions with cellular regulatory and signalling pathways. Importantly, taking into account the requirements for persistent virus infection in tumor cells, we suggest that treatment of HCMV-positive cancer patients with antiviral drugs abolishing HCMV-infection may be beneficial to suppress tumor progression.

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