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Detection of human cytomegalovirus in medulloblastomas reveals a potential therapeutic target

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Medulloblastomas are the most common malignant brain tumors in children. They express high levels of COX-2 and produce PGE2, which stimulates tumor cell proliferation. Human cytomegalovirus (HCMV) is prevalent in the human population and encodes proteins that provide immune evasion strategies and promote oncogenic transformation and oncomodulation. In particular, HCMV induces COX-2 expression; STAT3 phosphorylation; production of PGE2, vascular endothelial growth factor, and IL-6; and tumor formation in vivo. Here, we show that a large proportion of primary medulloblastomas and medulloblastoma cell lines are infected with HCMV and that COX-2 expression, along with PGE2 levels, in tumors is directly modulated by the virus. Our analysis indicated that both HCMV immediate-early proteins and late proteins are expressed in the majority of primary medulloblastomas. Remarkably, all of the human medulloblastoma cell lines that we analyzed contained HCMV DNA and RNA and expressed HCMV proteins at various levels in vitro. When engrafted into immunocompromised mice, human medulloblastoma cells induced expression of HCMV proteins. HCMV and COX-2 expression correlated in primary tumors, cell lines, and medulloblastoma xenografts. The antiviral drug valganciclovir and the specific COX-2 inhibitor celecoxib prevented HCMV replication in vitro and inhibited PGE2 production and reduced medulloblastoma tumor cell growth both in vitro and in vivo. Ganciclovir did not affect the growth of HCMV-negative tumor cell lines. These findings imply an important role for HCMV in medulloblastoma and suggest HCMV as a novel therapeutic target for this tumor.

Introduction

Medulloblastomas are molecularly and clinically diverse tumor types that together encompass the most frequent malignant brain tumors in children (1). Despite intensive multimodal therapy, survival in high-risk patients is still poor, and long-term survivors frequently experience physical and neuropsychological sequelae (2, 3), underscoring the need for new therapeutic options. Although medulloblastomas contain significantly fewer mutations than adult cancers (4), specific subsets have been identified, and aberrations of significant developmental pathways, including the Hedgehog and Wnt pathways, as well as frequent mutations of the MLL family genes have been described. However, since many medulloblastomas have no apparent mutations of any known cancer gene, it has been suggested that epigenetic changes also may be responsible for tumor initiation (4). Hence, additional etiological factors clearly connected to medulloblastoma development remain to be identified.

HCMV DNA and proteins are found in tumors of various origins, including 90%–100% of glioblastomas and prostate, breast, and colon cancers (5–7). This virus is not considered to be oncogenic; rather, its potential role in cancer seems to be oncomodulatory (8). Its 230-kbp genome has 252 open reading frames and encodes approximately 200 proteins, of which only about 50 are known to be essential for viral replication. Hence, the vast majority of HCMV proteins fulfill other functions in the viral life cycle and affect cellular and immunological functions relevant to tumor biology and immune surveillance. HCMV proteins control telomerase activity, cellular differentiation, proliferation (through specific effects on the cell cycle), apoptosis, epigenetic functions, migration, and angiogenesis (8–10). HCMV induces inflammation, but in parallel, it escapes immune recognition (8, 10). These tumor-promoting functions of HCMV proteins, together with the detection of HCMV DNA and proteins in tumors of different origins, suggest that HCMV has a pivotal, hitherto unknown role in the tumorigenesis of certain cancers (8).

The inflammatory mediator PGE2, a COX-2 metabolite implicated as a growth factor for tumor cells, drives cancer progression through its ability to stimulate cell proliferation, promote angiogenesis and invasion, inhibit apoptosis, and suppress immune responses (11–13). For unknown reasons, many tumors, including medulloblastomas, express COX-2, and inhibition of PGE2, synthesis with nonsteroidal antiinflammatory drugs induces apoptosis in medulloblastoma cell lines and significantly inhibits the growth...
of medulloblastomas in vivo (14). Aspirin reduces the incidence and growth of various cancer forms in animal models (15, 16), an effect that may be mediated at least in part by inhibition of COX-2 enzymes, resulting in decreased production of prostaglandins and other inflammatory mediators. In clinical trials, aspirin given daily to prevent vascular events for at least 4 years reduced death from different forms of cancer both during and after the trials (17). In clinical studies, nonspecific and specific COX-2 inhibitors have shown promise as anticancer agents (16, 18–20).

Interestingly, the HCMV protein US28, a constitutively active chemokine receptor homolog, induces COX-2 expression and results in STAT3 phosphorylation, which increases production of vascular endothelial growth factor and IL-6 as well as tumor formation in vivo (21). Both aspirin and selective COX-2 inhibitors efficiently prevent viral production, which is partly dependent on PGE$_2$ (22, 23). Transgenic mice that express US28 in intestinal epithelial cells exhibit a hyperplastic intestinal epithelium and tumor development. US28 expression in intestinal epithelial cells inhibits glycogen synthase–3β (GSK-3β) function, promotes accumulation of β-catenin, and increases expression of Wnt target genes involved in the control of cell proliferation (24); these findings provide a direct molecular link between this HCMV protein and oncogenesis. Moreover, we recently demonstrated that the HCMV protein IE72 induces telomerase activity, which is likely another important event in tumor transformation (25).

In this study, we examined the prevalence of HCMV in medulloblastomas. We also assessed the connection between HCMV and COX-2 expression in tumors and sought to determine whether inhibition of viral replication with antiviral treatment and COX-2 inhibition affects tumor growth in vitro and in vivo.

### Results

High prevalence of HCMV in primary medulloblastomas, medulloblastoma cell lines, and medulloblastoma xenografts. Of the 37 primary medullo-
We examined for HCMV, 34 (92%) expressed immediate-early (IE) proteins and 27 (73%) expressed late proteins (Table 1). The IE and late proteins were widely expressed in cells within the tumor (Figure 1), as determined by immunohistochemistry (n = 37, Figure 1, A, B, D, E, M, and N) or indirect immunofluorescence (n = 6, Figure 1, J, K, and L) in frozen tissue sections. Detection of IE proteins with two different IE-specific antibodies resulted in nuclear (Figure 1) or nuclear/cytoplasmic (see below) staining patterns. In situ hybridization confirmed the presence of HCMV in primary medulloblastomas (Figure 1, G–I). Flow cytometric analysis of these tumors revealed expression of HCMV IE and UL83 (pp65 in Figure 1O) proteins in 90%–95% of the cells (n = 2). Six available frozen tissue samples were confirmed to contain HCMV IE, pp150, and gB DNA and IE and gB RNA by TaqMan PCR (data not shown). Sequencing of the PCR products revealed that the HCMV genome in medulloblastoma cell lines was distinctly different from that in 4 commonly used laboratory strains (Merlin, AD169, TB40E, and VR1814; data not shown), which excluded the possibility of contamination.

Next, we analyzed 8 human medulloblastoma cell lines (D324 MED, D283 MED, UW228-3, MEB-MED-8A, D458 MED, PFSK-1, D384 MED, and D425 MED) for HCMV DNA and RNA by PCR. Surprisingly, HCMV IE and pp150 DNA and IE and gB RNA were detected at low levels in all 8 cell lines (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI57147DS1). FISH analyses with a probe utilizing the whole HCMV genome in bacterial artificial chromosome clones revealed HCMV DNA in medulloblastoma cells and in HCMV-infected (AD169) MRC-5 human fibroblasts, but not in K562 cells (control human chronic myelogenous leukemia cells; Figure 2A). To examine whether the viral genome was integrated in human chromosomes, we performed FISH analyses on colcemid-treated D324...
MED cells. No sign of integration of HCMV DNA in chromosomes was observed in metaphase cells (Figure 2A).

Further analysis by flow cytometry revealed HCMV IE and pp65 protein expression in 9%–39% of the cells; D324 MED cells stained for IE are shown in Figure 2B, and UW228-3 cells stained for IE and pp65 are shown in Figure 3B. On different sampling occasions over a 12-month period, HCMV proteins were expressed in 0.2%–75% of cells in individual medulloblastoma cell lines. Given this consistent presence of viral DNA, we hypothesized that HCMV resides in cancer stem cells. To test this hypothesis, we double stained the medulloblastoma cell line D283 MED for CD133 (a stem cell marker) and HCMV IE proteins. 57% of the CD133-positive cells coexpressed HCMV IE proteins (Figure 2C). When D283 MED cells were xenografted subcutaneously in NMRI nu/nu mice, HCMV protein expression distinctly increased, and a majority of the tumor cells were positive for HCMV IE proteins. While IE protein expression was diffuse in the xenografts, HCMV late protein expression was distinctly located adjacent to vessels (Figure 2D).

HCMV-positive medulloblastoma cells express COX-2. Since medulloblastomas express COX-2 and HCMV induces expression of COX-2 (14, 21), we investigated whether HCMV protein expression correlated with COX-2 expression in tumor cells. In frozen tissue sections of primary medulloblastomas, HCMV protein expression correlated with COX-2 expression (Figure 3A). IE proteins were expressed in both the nucleus and cytoplasm of tumor cells. In medulloblastoma cell lines, cells positive for the HCMV IE pp65 also expressed COX-2 (Figure 3B). HCMV and COX-2 expression also correlated in human medulloblastoma xenografts in mice (data not shown). These observations imply that HCMV may control the expression of COX-2 in medulloblastomas.

HCMV infection induces COX-2 expression and PGE₂ synthesis in medulloblastoma cells. HCMV induces COX-2 expression in fibroblasts and epithelial and smooth muscle cells (22, 23). We hypothesized that HCMV also regulates COX-2 expression in medulloblastomas. In vitro infection (superinfection) of medulloblastoma cells (D324 MED) with HCMV resulted in increased IE RNA expression at 3 hours after infection (relative mRNA expression, 0.04296, n = 3 in duplicate) that greatly increased at 6–72 hours after infection (Figure 4A). The late HCMV pp150 RNA was detectable at 3 hours after infection (relative mRNA expression, 0.00316, n = 3 in duplicates), and was greatly increased at 24 hours after infection (Figure 4A). Both IE and pp150 were detectable at low levels in the uninfected cells (mean relative mRNA expression, 0.0000322 and 0.00000132, respec-
natively; \( n = 3; 7 \) time points in duplicate). Interestingly, expression of COX-2 mRNA was already increased at 1 and 3 hours after infection in cells infected with both HCMV- and UV-treated virus inoculum (Figure 4A), before induced IE expression was detected. Also, an increased level of the COX-2 protein was detectable at 3 hours after infection (Figure 4B). As US28 has been demonstrated to induce COX-2 expression, we examined whether US28 was expressed at early time points after infection. We found that US28 mRNA could be detected in cells already at 15 minutes after infection: low US28 mRNA levels were also detected in UV-HCMV–infected cells. This was consistent with the increased expression of COX-2 mRNA observed in both HCMV- and UV-HCMV–infected cells at 1 and 3 hours after infection. At 3 days after infection, HCMV had significantly increased \( \text{PGE}_2 \) synthesis (Figure 4C), which was significantly reduced when D324 MED cells were treated with ganciclovir, celecoxib, or both drugs (Figure 4D); this was consistent with the detection of COX-2 mRNA in HCMV–infected but not UV-HCMV–infected cells at 72 hours after infection (Figure 4A).

**Antiviral treatment and COX-2 inhibition significantly inhibit medulloblastoma growth in vitro and in vivo.** Both specific and nonspecific COX-2 inhibitors can prevent virus-mediated accumulation of \( \text{PGE}_2 \) and efficiently inhibit viral production, as viral replication appears to be dependent on \( \text{PGE}_2 \) (22, 23). Therefore, we examined whether ganciclovir, which targets the DNA polymerase during HCMV replication, or the COX-2 inhibitor celecoxib, which prevents HCMV replication by decreasing \( \text{PGE}_2 \) levels, affects medulloblastoma growth in vitro.

In clonogenic assays with the native medulloblastoma cells, treatment with increasing concentrations of ganciclovir reduced the clonogenic capacity of D324 MED (\( P < 0.0001 \)), D283 MED (\( P < 0.0001 \)), and UW228-3 (\( P < 0.001 \)) (Figure 5A). Since none of the screened medulloblastoma cell lines were HCMV negative, we tested the effect of ganciclovir on the growth of two tumor cell lines that were negative for HCMV by PCR and flow cytometry (data not shown): PC-3 (established from a bone metastasis of a grade IV prostate adenocarcinoma) and BxPC-3 (established from a pancreatic adenocarcinoma). Treatment with increasing concentrations of ganciclovir had no significant effect on the clonogenic capacity of either cell line in the presence of ganciclovir (37.5, 75, or 150 \( \mu \text{M} \)) (Figure 5A).

Celecoxib also reduced the clonogenic capacity of D324 MED (\( P < 0.0001 \)) and D283 MED (\( P < 0.0001 \)) but not of UW228-3 (Figure 5B). In combination, valganciclovir and celecoxib significantly reduced the clonogenic capacity of medulloblastoma cell lines.
compared with single treatments alone (D324 MED, $P < 0.0001$; D283 MED, $P < 0.0001$; UW228-3, $P < 0.0001$; Figure 5B). To further test whether ganciclovir had a general cytotoxic effect, we performed survival assays (MTT) in human fibroblasts. At the same concentrations that significantly inhibited the growth of medulloblastoma cells, neither drug alone nor the combinations of the two drugs affected the survival of MRC-5 cells (Figure 5B). These data imply that the antitumor response in HCMV-positive medulloblastoma cell lines reflects a specific antitumor effect involving HCMV rather than cytotoxicity.

To further investigate whether the growth-suppressing effect of ganciclovir and celecoxib is dependent on the presence HCMV in medulloblastoma cells, we treated D283 MED cells with different concentrations of ganciclovir (37.5, 75, and 150 μM). After 2 weeks of continuous treatment, viral protein expression in cells treated with 75 μM ganciclovir had decreased substantially (data not shown and Figure 6A), but neither HCMV protein expression nor HCMV DNA was completely eliminated (Figure 6A). Although D283 MED cells pretreated with ganciclovir (D283 MEDgan) were able to grow in soft agar, they formed significantly fewer colonies than untreated D283 MED (D283 MEDwt) cells ($P < 0.05$; Figure 6A). The clonogenic capacity of D283 MEDgan cells was not affected by increasing concentrations of ganciclovir or celecoxib but was significantly reduced by the combination of the two drugs (Figure 6A).

In clonogenic assays with HCMV-superinfected medulloblastoma cells, treatment with increasing concentrations of ganciclovir or celecoxib also significantly reduced the tumorigenic capacity of HCMV-D324 MED, HCMV-D283 MED, and HCMV-UW228-3 cells in a dose-dependent manner (Figure 6B). Combining celecoxib with ganciclovir augmented the effect; growth was reduced by 81%, 84%, and 97% ($P < 0.0001$, Figure 6B), respectively, in superinfected cells. Consistent with these observations, valganciclovir and celecoxib alone or in combination also reduced PGE$_2$ production in D324 MED cells (Figure 4D).

To further investigate the therapeutic effects of valganciclovir and celecoxib in vivo, we established a human medulloblastoma xenograft model using D283 MED cells in NMRI nu/nu mice. We first determined the bioavailability of valganciclovir in these mice. After administration of valganciclovir at 100 mg/kg, the plasma ganciclovir concentration was sufficient for a potential therapeutic effect (Figure 7A). To observe a combinatorial effect with celecoxib, we reduced the valganciclovir dose. Mice were randomized to daily treatment for 12 days with oral valganciclovir (14 mg/kg twice daily) or oral celecoxib (90 mg/kg once daily), starting at a tumor volume of 0.1 ml. The mice were euthanized, and the tumors were collected for analysis. HCMV protein expression in the xenografts was strongly increased compared with that in the cells in culture (Figure 2D). Each drug inhibited the growth of established medulloblastomas by approximately 40% (valganciclovir, $P < 0.0001$; cele-
coxib, \( P < 0.0001 \); Figure 7, B and C). In combination, celecoxib and valganciclovir reduced tumor growth by 72% (tumor volume index [TVI], 3.3 versus 10.3 in untreated controls, \( P < 0.0001 \); Figure 7, B and C). The combined drug regimen reduced the number of clusters positive for HCMV late protein in xenograft tumors by 80% (\( P < 0.0001 \); Figure 7, D and E).

We also established xenografts of the two HCMV-negative tumor cell lines (PC-3 and BxPC-3) to test the effect of ganciclovir on tumor cell growth. In NMRI \( nu/nu \) mice carrying either PC-3 or BxPC-3 xenografts, tumor growth was unaffected by treatment with oral valganciclovir (14 mg/kg twice daily) (tumor weight: PC-3, \( P = 0.4810 \); BxPC-3, \( P = 0.7237 \); Figure 7, F and G).

**Discussion**

This study shows that HCMV proteins are expressed in the majority of primary medulloblastoma tissue samples. Surprisingly, HCMV DNA, RNA, and proteins were detected in 8 examined medulloblastoma cell lines, and viral protein expression was highly induced by xenografting of human medulloblastoma cells in vivo. Sequencing of PCR products excluded the possibility that the cells lines were contaminated by a laboratory strain. Targeting of HCMV by antiviral or COX-2–inhibitory drugs with different modes of action on viral replication significantly impaired tumor growth in vivo. These observations imply a persistent source of HCMV in neuroprecursor cells and suggest that the virus has a pathogenetic role in medulloblastoma rather than representing an epiphenomenon. Importantly, our findings also suggest HCMV as a novel therapeutic target for this tumor.

Medulloblastomas can originate from neural precursor cells in the external granule layer in the cerebellum or from cells in the dorsal brainstem (26). Medulloblastoma cells exhibit many characteristics similar to those of progenitor cells of the embryonic brain, further suggesting the presence of a tumor-initiating or cancer stem cell population in these tumors (26). This statement is supported by the clinical presentation and treatment response and by the fact that cells positive for stem cell markers (e.g., CD133 or CD15 in primary medulloblastomas) give rise to new tumors in xenograft models (27, 28). Since HCMV appears to persist for years in laboratory cell lines, the virus is most likely maintained in a stem-like cell phenotype within this population. Indeed, we observed HCMV protein expression...
in 57% of CD133-positive D283 MED cells. The viral genome did not appear to be integrated into human chromosomes, and we concluded that HCMV integration evidently is not a major event in the medulloblastomas, although it cannot be excluded in single cells.

HCMV can infect neuronal progenitor cells and is the major infectious cause of birth defects in newborns, including sensorineural hearing loss or neuronal migration disturbances during brain development and, in the most severe cases, microcephaly or anencephaly. Previously, we showed that infection of neuronal progenitor cells blocks their ability to differentiate into neurons (29) or astrocytes (30). Interestingly, prevention of the normal differentiation of neuronal stem cells has been implicated as an important step in the early development of brain tumors (31). We speculate that HCMV infection of a defined tumor precursor cell is a decisive early event in the disordered cerebellar development that leads to medulloblastoma (32) through HCMV-spe-
Ganciclovir and celecoxib significantly impair the growth of established human medulloblastoma xenografts in NMRI nu/nu mice. (A) Pharmacokinetics of valganciclovir (val) (plasma concentration versus time) after 1 oral dose (100 mg/kg). Solid line represents concentration-time data estimated by pharmacokinetic modeling. NMRI nu/nu mice were engrafted with $7 \times 10^6$ D283 MED cells subcutaneously and randomized to receive 14 mg/kg valganciclovir twice daily, 90 mg/kg celecoxib daily, both drugs, or no treatment. All treatments were given orally through a gastric feeding tube for 12 days, starting when tumors were 0.1 ml (mean, 0.13 ml). (B and C) Valganciclovir augments the inhibitory effect of celecoxib on medulloblastoma growth in vivo, as shown by the TVI (B) and tumor weight (C) at autopsy. TVI values are mean ± SD; tumor weight is shown as median and quartiles (TVI, 2-tailed ANOVA; tumor weight, 1-way ANOVA). (D and E) Clusters of cells positive for HCMV late protein in xenograft tissues were reduced by 80% by valganciclovir and celecoxib treatment. Untreated ($n = 5$), 75 ± 30; valganciclovir + celecoxib ($n = 7$), 15 ± 5. Values are mean ± SD. **P < 0.0001 (t test). NMRI nu/nu mice were also engrafted subcutaneously with the HCMV-negative cell lines PC-3 ($30 \times 10^6$ cells) and BxPC-3 ($5 \times 10^6$ cells) and randomized to receive 14 mg/kg valganciclovir by gastric feeding twice daily or no treatment for 23 days (PC-3) or 32 days (BxPC-3). (F and G) PC-3 or BxPC-3 xenograft growth was not inhibited in mice treated with ganciclovir, as shown by the TVI (F) and tumor weight (G) at autopsy. TVI values are mean ± SD; tumor weight is shown as median.
specific effects on tumorigenesis, inflammation, and immune evasion strategies. HCMV infection and expression of US28 result in STAT3 phosphorylation, Wnt activation, enhanced COX-2 expression, production of vascular endothelial growth factor, increased production of PGE₂ and IL-6, and inflammation. Transgenic mice (VS28 mice) with US28 expression targeted to intestinal epithelial cells exhibit a hyperplastic intestinal epithelium and develop adenomas and adenocarcinomas at 40 weeks of age (24). When exposed to inflammation, VS28 mice have a significantly higher tumor burden (24). These observations imply that US28 by itself promotes dysplasia and cancer development, especially during enhanced inflammation. HCMV induces inflammation both by its presence as a foreign antigen in tissues and by inducing expression of COX-2 and 5-lipooxygenase, which results in production of prostanoids and leukotrienes (33). Here, we observed COX-2 expression in HCMV protein–positive cells in primary medulloblastoma samples and in medulloblastoma cell lines, and the induction of COX-2 expression and PGE₂ production in experimentally infected medulloblastoma cells. US28 mRNA was detected at 15 minutes and at 1 and 3 hours after infection and before the detection of HCMV IE RNA. It is likely that US28 RNA was delivered with the viral particle, a phenomenon that is known to occur during HCMV infection of tumor suppressor proteins will promote cell cycle progression and increase DNA synthesis while simultaneously elongating HCMV IE RNA. It is likely that US28 RNA was delivered with the telomeres and blocking apoptosis (39–42). In primary glioblastoma cells, we found that mainly HCMV IE–positive cells expressed hTERT, and the HCMV IE72 protein directly interacted with the hTERT promoter at Sp1 binding sites to induce telomerase activity and telomere elongation (25). This scenario will most likely be important in tumor transformation.

The two HCMV IE–specific antibodies we used to detect IE proteins in primary medulloblastoma tissue specimens resulted in different staining patterns. The CH160 clone from Abcam recognizes both IE72 and IE86, while the Chemicon antibody recognizes a 68- to 72-kDa protein, which likely explains the different staining pattern of IE protein expression revealed by direct immunofluorescence and immunohistochemistry. We and others have noted that IE protein is expressed in both the cytoplasm and the nucleus in tumor cells, a pattern that may depend on different IE splicing variants encoded by the IE gene (43).

At the same time as HCMV induces inflammation and may affect tumorigenesis, many HCMV proteins use sophisticated mechanisms that make the infected cells invisible to the immune system. The result is enhanced viral replication and tumor growth. In support of this statement, the viral load in glioblastomas appears to be associated with patient outcome. We recently observed significantly longer median survival in glioblastoma patients with low-grade HCMV infection in the tumor than those with high-grade infections. The 2-year survival rate was also greater in patients with low-grade infections. These findings imply that the HCMV infection level in glioblastomas has a high prognostic value and that HCMV may contribute to the pathogenesis of glioblastoma rather than representing an epiphenomenon of the disease (our unpublished observations).

Targeting HCMV may be a new therapeutic option for medulloblastoma patients. In medulloblastoma cell lines, treatment with ganciclovir markedly reduced the number of HCMV-positive cells but did not eliminate the virus. Furthermore, treatment of medulloblastoma cells with two drugs with different modes of action efficiently inhibited tumor growth in vitro and in vivo. In combination, the drugs reduced tumor growth by 81%–97% in clonogenic assays and by 72% in NMRI nu/nu mice carrying established human medulloblastoma xenographs. In sharp contrast, ganciclovir had no effect on the clonogenic capacity or tumor growth of two HCMV-negative cell lines (PC-3 and BxPC-3) in vitro or in vivo. Importantly, ganciclovir had no significant toxic or antiproliferative effect on non-tumor cells that were HCMV negative.

These observations imply that the inhibitory effect of ganciclovir on the growth of medulloblastoma cell lines and tumors is HCMV specific and is not mediated by potential nonspecific drug effects on cellular proliferation. Since COX-2 inhibitors efficiently prevent HCMV replication (22, 44) and reduce the growth of US28-expressing tumor cells (21, 45), the ability of COX-2 inhibitors to protect against cancer in clinical trials may be mediated by control of HCMV replication. Since HCMV has strong oncomodulatory effects, treatment to control HCMV infection in tumor tissues may suppress tumor progression, improve the patient’s immune response, and increase the tumor’s sensitivity to conventional therapy.

Antiviral drugs for HCMV and COX-2 inhibitors are well tolerated and may be a new therapeutic option to be used in combination with conventional therapies in patients carrying HCMV-infected tumors. Indeed, we are evaluating antiviral drugs against HCMV as an adjuvant therapy for glioblastoma in a double-blind, placebo-controlled pilot study of 42 patients.

In summary, our observations suggest that HCMV is a novel etiological factor linked to medulloblastoma development and that nontoxic antiinflammatory and antiviral drugs targeting HCMV may be used in addition to conventional chemotherapy to treat patients with medulloblastoma.

**Methods**

**Cell cultures and HCMV.** The origin of the cells, culture conditions, and viability assays of human medulloblastoma and supratentorial primitive neuroectodermal cell lines have been described (14). Human prostate cells,
pancreas adenocarcinoma cells, PC-3 and BxPC-3 cells, human fibroblasts (MRC-5), and KS62 cells (human chronic myelogenous leukemia) were grown in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, 100 IU/ml penicillin G, and 100 μg/ml streptomycin (Life Technologies). Cells were grown to approximately 50–60% confluence and infected in low-serum medium for the indicated time with HCMV clinical isolate–like strain VR1814 at an MOI of 10. Controls were either not exposed to virus or were exposed to UV-inactivated virus (UV Stratalinker 1800; Stratagene). HCMV VR1814 (provided by G. Gerna, University of Pavia, Pavia, Italy) was prepared from supernatants of cultures of in vitro infected human umbilical vein endothelial cells, grown in EGM2 medium (Clonetics, Cambrex), frozen, and stored at −20°C.

The diagnosis of medulloblastoma was confirmed by his

0.1% Tween 80 (v/v; Sigma-Aldrich) in sterile water.

were incubated with the primary antibodies for 60 minutes at room temperature. The primary antibodies were mouse monoclonal IgG1 isotype control (5 μg/ml, Acris Antibodies). Sections were rinsed in PBS containing 0.1% saponin and incubated with the secondary antibodies for 30 minutes at room temperature in the dark. The secondary antibodies were goat anti-mouse Alexa Fluor 594 (5 μg/ml, Molecular Probes, Invitrogen). Sections were then rinsed in PBS and mounted wet in ProLong Gold Antifade medium (Molecular Probes, Invitrogen) containing DAPI for nuclear staining. Staining for IE and late proteins was evaluated by grading sections as negative or from 1–4, depending on the estimated percentage of positive cells: 1: <25%, 2: 25%–50%, 3: >50%–75%, 4: >75%.

Flow cytometry. Resected tumor material was placed in sterile 50-ml tubes containing cell culture medium (UltraCulture, Lonza BioWhittaker-VWR) supplemented with 2 mM l-glutamine (Lonza BioWhittaker-VWR), referred to as UC, at the surgical theater and transported on ice to the cell culture facility. One piece of tumor was cryopreserved for sectioning, and one piece was dissociated into a single-cell suspension for flow cytometric analysis.

For single-cell suspensions, the tumor was cut into small pieces with a sterile scalpel blade in a petri dish containing Accutase (Sigma-Aldrich). The tumor was incubated for 15 minutes at 37°C. During this time, the tumor was removed once from the incubator and dissociated through a 10-ml serological pipette. After incubation, the tumor was further dissociated by pipetting and transferred to a cell strainer (75 μm) (Falcon, BD Biosciences — Pharamingen) on a 50-ml tube. A 2-ml syringe plunger was used to mince the tumor through the cell strainer. Approximately 8 ml UC was used to rinse the strainer. Cells were centrifuged for 8 minutes at 335 g, resuspended in UC, and counted.

To permeabilize cells for intracellular staining, the Cytofix/Cytoperm kit (BD) was used according to the manufacturer's instructions. Cells were washed twice in PBS without Ca²⁺ and Mg²⁺ (Gibco, Invitrogen) supplemented with 1% bovine serum albumin fraction V (Roche) before the primary antibody was added (mouse monoclonal [CH12] to cytomegalovirus UL83, Abcam) for 60 minutes at 4°C. Cells were washed as above before incubation with the secondary antibody, FITC-conjugated anti-mouse IgG (eBioscience), for 30 minutes at 4°C and washed twice in PBS and 1% bovine serum albumin fraction V. As negative control, the primary antibody was omitted, and cells were incubated with the secondary antibody only. Cells were analyzed with a flow cytometer (C6, Accuri). Gating for tumor cells was based on forward and side scatter, and data were analyzed with CFlow Plus software (Accuri).

The medulloblastoma cell lines (D324 MED, D283 MED, and UW228-3) were permeabilized with the Perm 2 kit (BD) according to the manufacturer’s instructions. The following antibodies were used: mouse monoclonal IgG1 (Dako), IE antigen (mouse monoclonal 11-003, Argene), pp65 (mouse monoclonal, Novocastra), MAB810R (mouse monoclonal, Chemicon), and COX-2 (rabbit polyclonal, Santa Cruz Biotechnology Inc.). The cells were incubated with the primary antibody for 30 minutes at 4°C, washed with PBS, and incubated for 30 minutes at 4°C with the secondary antibody (FITC-conjugated polyclonal rabbit anti-mouse IgG, Dako). For double staining for COX-2 and IE antigen (Argene), cells were incubated with the secondary antibodies FITC-conjugated swine anti-rabbit (Dako) and R-PE–conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories Inc.). After incubation, cells were washed with PBS, fixed with 1% paraformaldehyde, and analyzed with a CyAn flow cytometer (Beckman Coulter) and Summit 4.3 software.

DNA extraction and HCMV PCR. DNA was extracted from cell pellets with the QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer’s protocol. HCMV IE and pp150 genes were detected by real-time PCR (Invitrogen, Life Technologies) with specific primers (IE: GTGACCCATGTGCTTATGCCTCTAT, CTCAACATAGTCGCAAGAACGT, FAM-TTGGTCACGGGTGTCTC; pp150: GCGCCGGGAACCTCCT, CCGT-
GGCGCGACAAAAACG, FAM-CAGCGGTCAAGCCTCG). The housekeeping gene RnasP served as a control (Applied Biosystems).

Quantitative PCR. D324 MED cells were seeded in 6-well plates (Sarstedt) at 1 × 10^4 cells/well and superinfected with HCMV VR1814 (MOI of 10) in low-serum medium. Cells were collected 15 minutes and 1, 3, 6, 24, 48, and 72 hours after infection and frozen at −80°C before RNA extraction with the RNeasy RNA kit (Qiagen). cDNA was synthesized from equal amounts of RNA with the SuperScript III First-Strand Synthesis System (Invitrogen, Life Technologies). cDNA was then used for quantitative PCR for HCMV IE, pp150, US28, and COX-2 with TaqMan gene expression assays (Applied Biosystems). RnasP, β-2 microglobulin, and β-actin (Applied Biosystems) served as controls. Each experiment was performed in duplicate and repeated 3 times.

In situ hybridization. HCMV DNA in paraffin sections was detected as described previously (5) with minor modifications. Fluorescein-labeled HCMV total genome DNA probe (Zymed, Invitrogen) was denatured on paraffin sections with a MISHA thermocycler (Shandon Lipshaw), and slides were hybridized overnight at 37°C in a humidified chamber. Specific endogenous Alu DNA sequence and nonspecific DNA-labeled control fluorescein probes provided by the manufacture (Zymed, Invitrogen) served as positive and negative controls, respectively. Probes were detected by using alkaline phosphate–labeled antibodies against fluorescein (Zymed, Invitrogen) and Fast Red chromogen (BioGenex).

Immunoblotting. Total cell protein lysates were extracted from cells in RIPA buffer (25 mM Tris, pH 7.8, 2 mM EDTA, 20% glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol). All protein extraction buffers were supplemented with Mini Complete protease inhibitor cocktail (Roche Diagnostics), 1 mM NaF, and 1 mM Na2VO4. Protein concentration was measured with the Bradford reagent (Bio-Rad). Equal quantities were separated by SDS-PAGE, transferred to nylon membranes (Millipore), and probed with antibodies against HCMV IE (Argene; 1:200), COX-2 (Santa Cruz Biotechnology Inc.; 1:500), and β-actin (Sigma-Aldrich; 1:1000). The secondary antibodies were anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase (GE Healthcare). Pierce SuperSignal was used to detect chemiluminescence.


Statistics. All statistical analyses were performed with GraphPad Prism software (GraphPad Software). For in vitro studies, the t test (2-tailed) was used to determine whether the mean of a single sample differed significantly from control. To compare several treatment groups, I-way ANOVA with Tukey multiple-comparisons tests were used. A P value less than 0.05 was considered significant. Tumor growth was analyzed by unpaired, 2-tailed ANOVA and tumor weight by 1-way ANOVA; for both, the Bonferroni post hoc test was used for multiple comparisons between groups.

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