5-Azacytidine treatment results in nuclear exclusion of DNA methyltransferase-1, as well as reduced proliferation and invasion in human cytomegalovirus-infected glioblastoma cells

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Abstract. Glioblastoma (GBM) is the most aggressive form of brain tumor in adults, with a devastating outcome. Emerging evidence shows that human cytomegalovirus (HCMV) proteins and nucleic acids are present in GBM tissues. DNA methylation is important for the initiation and progression of cancer and is an established host response against invading nucleic acids. The expression and localization of DNA methyltransferase 1 (DNMT-1) was assessed, and the effects of DNA methylation inhibitor 5-azacytidine (5AZA) were analyzed in the context of the viral replication, proliferation and invasion capacities of HCMV-infected GBM U343MG cells. In addition, the expression of various HCMV proteins and DNMT-1 was examined in GBM tissue specimens obtained from five patients. DNMT-1 was localized in the nucleus of cells expressing HCMV-immediate early, whereas in cells expressing HCMV-glycoprotein gB (gB), extranuclear/cytoplasmic localization was observed. This was also observed *in vitro* in U343MG cells. In addition, DNMT-1 was localized to the extranuclear/cytoplasmic space of cells lining blood vessel walls within the GBM tumors. Treatment of infected U343MG cells with 5AZA did not affect viral replication, but reduced cell invasion and proliferation (P=0.05 and P<0.0001, respectively). However, 5AZA treatment of uninfected cells did not affect cell invasion (P=0.09), but proliferation was significantly reduced (P<0.0001). These findings may be of importance in further investigations aimed at using DNA methylation and viral inhibitors in GBM therapy.

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Introduction

Human cytomegalovirus (HCMV) is a member of the *Herpesviridae* family with a genome size of 236 kb, the largest of any known human virus (1,2). The HCMV genome encodes 750 proteins, of which ~50 are involved in the construction of new viral particles. The vast majority of viral proteins function in regulating important host functions that assist the virus to co-exist with its host (3). During evolution, HCMV has developed unique mechanisms to adapt to the human immune system, allowing it to maintain a latent phase in $CD34^+$ myeloid progenitor cells (4). Originally it was thought that HCMV is completely inactive during latency. However, various viral transcripts and proteins appear to be induced during this phase, including latency unique natural antigen (LUNA) (5), G-protein coupled receptor homolog US28 (US28), viral interleukin (IL)-10 homolog (UL111A) and membrane glycoprotein UL144 (UL144) (6). These viral proteins affect the immune system. For example, LUNA stimulates the immune response (5), US28 is associated with inflammation, and UL111A and UL144 are involved in HCMV immune evasion (7). Furthermore, different biological conditions were shown to be central in the reactivation of latent HCMV, including inflammation, immune suppression, other infections and epigenetic modifications (8,9). The critical factor in transcriptional reactivation of latent HCMV is terminal differentiation of latently infected monocytes into macrophages or dendritic cells (8,10). Accumulating evidence suggests an association between histone modification, chromatin modulation, HCMV genome

transcription and replication, both during latency and lytic infection (9,11,12). Additionally, previous studies have shown that histone deacetylases (HDACs) are bound to the HCMV promoter and HDAC activity results in repression of viral gene expression and latency (13-15). However, the involvement of host cell epigenetic alterations in HCMV reactivation remains poorly understood.

Previous studies have revealed the frequent presence of HCMV nucleic acid sequences and proteins in malignant brain tumors, including glioblastoma multiforme (GBM), medulloblastoma (MB) and neuroblastoma (16-18). GBM is the most aggressive brain tumor in adults, with fatal outcomes. Despite aggressive surgery and advanced therapy, the median overall survival of these patients is ~14 months (19). The risk factors for GBM are unknown but environmental exposures to radiation, vinyl chloride and pesticides have been considered in the initiation of GBM (20,21). Traumatic brain injury and consequent inflammation have also been suggested in the etiology of GBM, although this is still controversial (22).

HCMV has not been classified as an oncogenic virus as it does not have the ability to transform cells, but the term oncomodulation has been used to describe the actions of HCMV in tumors (23). HCMV may modulate cancer progression through interactions with potential oncogenic factors within the host cells, such as p53, retinoblastoma protein (Rb) and cyclins, resulting in uncontrolled cell proliferation (24). Our recent report found that HCMV replication significantly increased in HCMV infected MB cells treated with 5-azacytidine (5AZA) (25). Since epigenetic drugs are suggested as agents of cancer therapy (26) and GBM tissues have been shown to be frequently positive for HCMV proteins and nucleic acids, the present study investigated the viral replication, proliferation and invasion capacity of 5AZA-treated HCMV-infected GBM cells (U343MG), and examined the expression of DNMT-1 and HCMV proteins in GBM tissue specimens.

Materials and methods

Cell culture and HCMV infection. U343MG and MRC-5 cells were obtained from American Type Culture Collection (Manassas, VA, USA). All cells (1.5-3.0x10^5) were cultured under the following conditions, unless otherwise stated: Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a humidified 5% CO_2. Cells were infected with HCMV clinical isolate (strain VR1814; a kind gift from Dr Giuseppe Gerna, University of Pavia, Pavia, Italy) at a multiplicity of infection (MOI) of 5 for U343MG cells and a MOI of 1 for MRC-5 cells, for 3 days. In all experiments, cells cultured without HCMV were used as the negative control. Transcripts from HCMV infected MRC-5 cells were used as positive control [with an infection efficiency for HCMV-Major Immediate-Early gene equal to C_t=21.2 from quantitative TaqMan polymerase chain reaction (qPCR); protocol described below], since MRC-5 cells are permissive for infection using the HCMV strain VR1814 (>80% infected cells). The number of cells used in different treatment experiments was 1.5-3.0x10^5. All experiments were performed with three independent repeats.

Immunofluorescence staining. Uninfected and HCMV infected U343MG cells (1.5-3.0x10^5) were fixed in ice-cold acetone:methanol (1:1) for 10 min (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Immunofluorescence staining of different proteins was performed as described previously, with minor modifications (25). Endogenous non-specific binding was blocked using Protein Block, Serum-Free (cat. no. X0909; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). The cells were incubated with the following primary antibodies: Mouse monoclonal antibody to immediate early (IE-1 and IE-2; 1:100; cat. no. 11-003; ARGENE; bioMérieux, Verniolle, France); mouse monoclonal antibody to glycoprotein gB (gB; 1:50; cat. no. P1216; EastCoast Bio, Inc., North Berwick, ME, USA); rabbit polyclonal antibody to DNMT-1 (1:500; cat. no. ab19905; Abcam). Binding of primary antibodies to specific proteins was detected by incubation with Alexa Fluor 488-conjugated goat anti-mouse (1:500; cat no: A-11001; Molecular Probes; Thermo Fisher Scientific, Inc.) and Texas red-conjugated goat anti-rabbit (1:500, cat no: T-6391, Molecular Probes; Thermo Fisher Scientific, Inc.) as secondary antibodies for 45 min at room temperature. Incubation with PBS instead of primary antibodies served as negative control. Ready to use fluorescence mounting medium (with DAPI; Vectashield; Vector Laboratories, Inc., Burlingame, CA, USA) was used for 1 min for nuclear staining and mounting. Staining was evaluated by confocal microscopy (scale bar, 50 μm; Leica TCS SP5; Leica Microsystems GmbH, Wetzlar, Germany).

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). A RNeasy kit (Qiagen AB, Sollentuna, Sweden) was used to extract the total RNA from cells according to the manufacturer's instructions. The RNA concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). RNA (500 ng) was converted to cDNA using the SuperScript III First-Strand Synthesis system (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. TaqMan qPCR with specific target primers and probes (FAM fluorophore) was performed using a 7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Target primers and probes used in this study were as follows, as described previously (25): HCMV-IE (forward primer, 5'-TGACGAGGGCCC TTCCT-3' and reverse primer, 3'-CCTTGGTCACGGGTG TCT-5'; probes, FAM-AAGGTGCCACGGCCCG-NFQ) and HCMV-gB (forward primer, 5'-GCTACCGCCCTACCTCAA G-3' and reverse primer, 3'-CGCCAACGGCCTTTCC-5'; probes, FAM-CCCAGGCCGCTCATG-NFQ) and DNMT-1 (assay ID, Hs00945875_m1; Life Technologies; Thermo Fisher Scientific, Inc.). RNase P (housekeeping gene; assay ID, 4316844; Life Technologies; Thermo Fisher Scientific, Inc.) was used for normalization. All assays were performed according to the manufacturer's protocols.

The qPCR thermocycler was initiated with polymerase activation at 95˚C for 20 sec followed by 40 cycles of denaturation at 95˚C for 1 sec and annealing/extension at 60˚C for 20 sec. PCR results were analyzed with SDS version 2.4 software (Thermo Fisher Scientific, Inc.). The ΔCt method was used for calculation of Ct values for different transcripts. The $2^{-\Delta\Delta Ct}$ method was used to quantify relative fold changes (27).

Table I. GBM patient characteristics.

Patient	GBM	Sex	Age	P53 mutations	KI-67 (%)	TTP (months)	OS (months)
1	Primary	Female	62	Negative	80	NR	3
2	Primary[a]	Male	53	ND	25	8	14.5
3	Primary	Male	75	Positive	25	3	11
4	Primary	Male	62	Negative	35	9	15.5
5	Secondary[b]	Female	34	Positive	70	NR	38[c], 8[d]

[a]Oligodendrogliomas; [b]GBM progressed from astrocytoma grade II; [c]time until secondary GBM occurrence; [d]OS following secondary GBM. GBM, glioblastoma multiforme; ND, not determined; NR, not relevant; TTP, time to tumor progression.

5AZA treatment. U343MG cells were treated with 5AZA (5-Aza-2'-deoxycytidine; 10 μM; Sigma-Aldrich; Merck KGaA) for 3 days and subsequently infected with HCMV-VR1814 at a MOI of 5 for 3 days, whilst maintaining the 5AZA concentration. Cells were fixed in ice-cold acetone:methanol (1:1) for 10 min 3 days post-infection (dpi) and were kept at -20˚C until further experimentation.

Ganciclovir® treatment. Ganciclovir® (2 mM; cymevene; Apoteket AB, Stockholm, Sweden) was used to treat U343MG cells at the time of HCMV infection. Cells were fixed as described above.

Invasion assay. Cells were plated on 6-well plates (day 0), untreated ($2x10^5$) or treated with 5-AZA ($3x10^5$) from days 0-5. On day 2, cells were infected (or not) with HCMV (MOI of 5). On day 5, cells were detached from the plates and used for the invasion assay. Invasion assays were performed using CytoSelect™ 6-Well Cell Migration and Invasion assay kit (8 μm; Colorimetric Format; Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's instructions. Briefly, under sterile conditions, untreated or 5AZA treated uninfected or HCMV infected cells in serum free culturing medium were plated on each insert and culturing media containing 10% FBS was added in the lower well of the migration plate. The plates were incubated at 37˚C (5% CO_2) for 24 h. Non-migrated cells on the upper side of the inserts were gently removed by wet cotton-tipped swabs. The inserts were transferred to a new wells and 200 μl extraction solution (provided in the kit) was added followed by a 10 min incubation on an orbital shaker. Finally, 100 μl from each sample was transferred to a 96-well microtiter plate. The optical density of the resulting extracts was measured at λ=560 nm and normalization was performed with the proliferation values at time 0 before treatment.

Proliferation assay. Uninfected and HCMV infected U343MG cells, untreated or treated with 5AZA, were seeded in 96-well culture plates (1,000/well) and allowed to attach. At 3 days post-treatment, cell viability was assessed using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation assay (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The absorbance was recorded at λ=490 and normalization was performed with the proliferation values at day 2, prior to treatment.

Immunohistochemical staining (IHC). Paraffin embedded human GBM tumor tissue sections from five patients (four patients with primary and one patient with secondary GBM; one primary GBM patient had chromosome 1p19q deletion; Table I) were retrospectively and randomly obtained between October 2009 and November 2010 from the Department of Pathology at Karolinska University Hospital (Stockholm, Sweden). The inclusion criterion was GBM tumor tissue. No recruitment period or other control samples/groups were defined. GBM tumor tissue samples were obtained at surgery at Karolinska University Hospital from adult GBM patients with written informed consent. Samples were stored in a biorepository and anonymized. Ethical permission was obtained from the Ethics Committee at the Karolinska Institutet (Stockholm, Sweden; Dnr. 2008/628-31).

Immunohistochemical staining of the tissues was performed as described previously, with some minor modifications (16,17). Following deparaffinization of the tissue specimens in xylene and rehydration in a series of decreasing ethanol concentrations, tissue sections were permeabilized with pepsin (Nordic BioSite AB, Täby, Sweden) and citrate buffer (Bio-Genex Laboratories, Fremont, CA, USA). Endogenous peroxidase activity was blocked with 3% H_2O_2 (Sigma-Aldrich; Merck KGaA) and the sections were treated with an avidin/biotin blocking kit (cat. no. X0590; Dako; Agilent Technologies, Inc.), Fc receptor blocker (cat. no. NB309; Innovex Biosciences, Richmond, CA, USA) and Background Buster (cat. no. NB306; Innovex Biosciences) to eliminate non-specific binding. All blocking steps were performed according to the kit protocols. ImmPRESS reagent kits (cat. no: MP-7401 and MP-7402; Vector Laboratories, Inc.) and diaminobenzidine were used to detect binding of primary antibodies to targeted proteins. The primary antibodies used in IHC were as follows: HCMV-IE (cat. no. MAB810R; Merck KGaA), HCMV-Late (cat. no. MAB8127; Merck KGaA), HCMV-gB (a kind gift from Dr William Britt, University of Alabama, USA), DNMT-1 (cat. no. ab19905, Abcam). For the negative control group, primary antibodies were omitted. For scanning of IHC sections, a Hamamatsu Nano Zoomer-XR Digital slide scanner C12000 (Hamamatsu Phototonics, Hamamatsu, Japan) was employed, with visualization using the Nano Zoomer Digital Pathology (NDP) viewer software (U12388-01; NDP.view2 Viewing; Hamamatsu Phototonics).

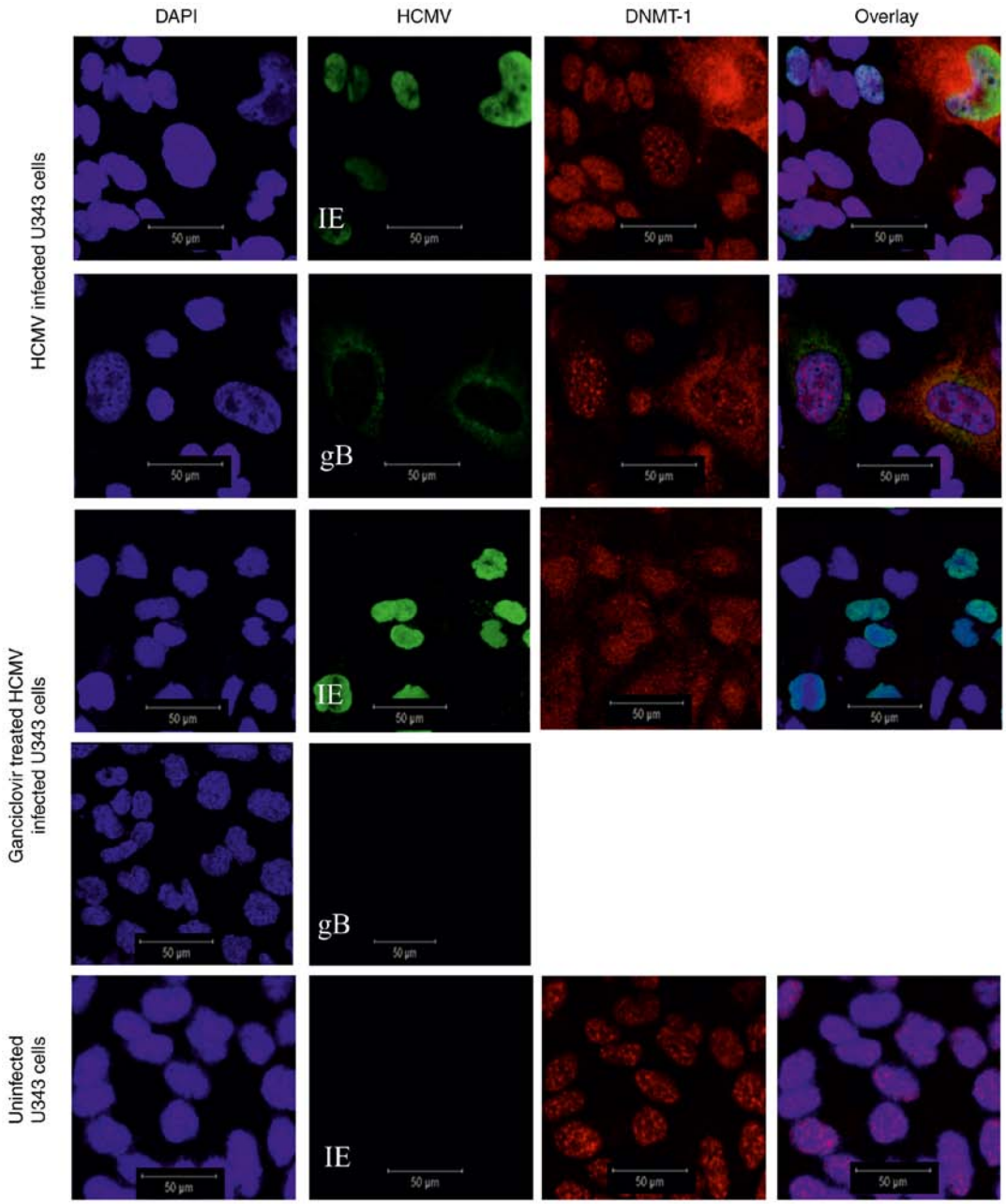

Figure 1. Cytoplasmic expression of DNMT-1 in HCMV-infected U343MG GBM cells expressing HCMV-gB protein. Expression of DNMT-1 in the nucleus of HCMV-IE positive cells and in the cytoplasm of HCMV-gB expressing U343MG GBM cells. DNMT-1 was expressed in the nucleus of Ganciclovir®-treated HCMV-infected cells expressing HCMV-IE, but not HCMV-gB proteins (scale bar, 50 μm). DNMT-1, DNA methyltransferase 1; HCMV, human cytomegalovirus; GBM, glioblastoma; gB, glycoprotein gB; IE, immediate early.

Statistical analysis. All analyses were performed using GraphPad Prism version 6 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference. Unpaired Student's t-test or one-way analysis of variance followed by Dunnett's multiple comparisons test were used to assess the statistical significance between different variables. Data are presented as the mean ± standard error of the mean. All experiments were performed with three independent repeats.

Results

Cytoplasmic expression of DNMT-1 in HCMV infected U343MG cells expressing viral late genes. U343MG GBM cells were infected with HCMV for 3 days in order to observe full length viral replication. Expression of DNMT-1 was detected in the nucleus of uninfected and HCMV-IE expressing cells (i.e. cells displaying immediate early phases of HCMV infection), but was evidently expressed exclusively in the extranuclear space of HCMV-gB positive cells (i.e. cells displaying later phases of HCMV infection) (Fig. 1). At 3 dpi, 18 and 4% of the U343MG cells expressed HCMV-IE and HCMV-gB proteins, respectively, ($P=0.002$) and the HCMV-IE transcript was present at higher levels than HCMV-gB transcripts in the infected cells ($P=0.0007$; Fig. 2A and B). DNMT-1 transcript levels did not change significantly in HCMV-infected cells compared to uninfected cells (Fig. 2C).

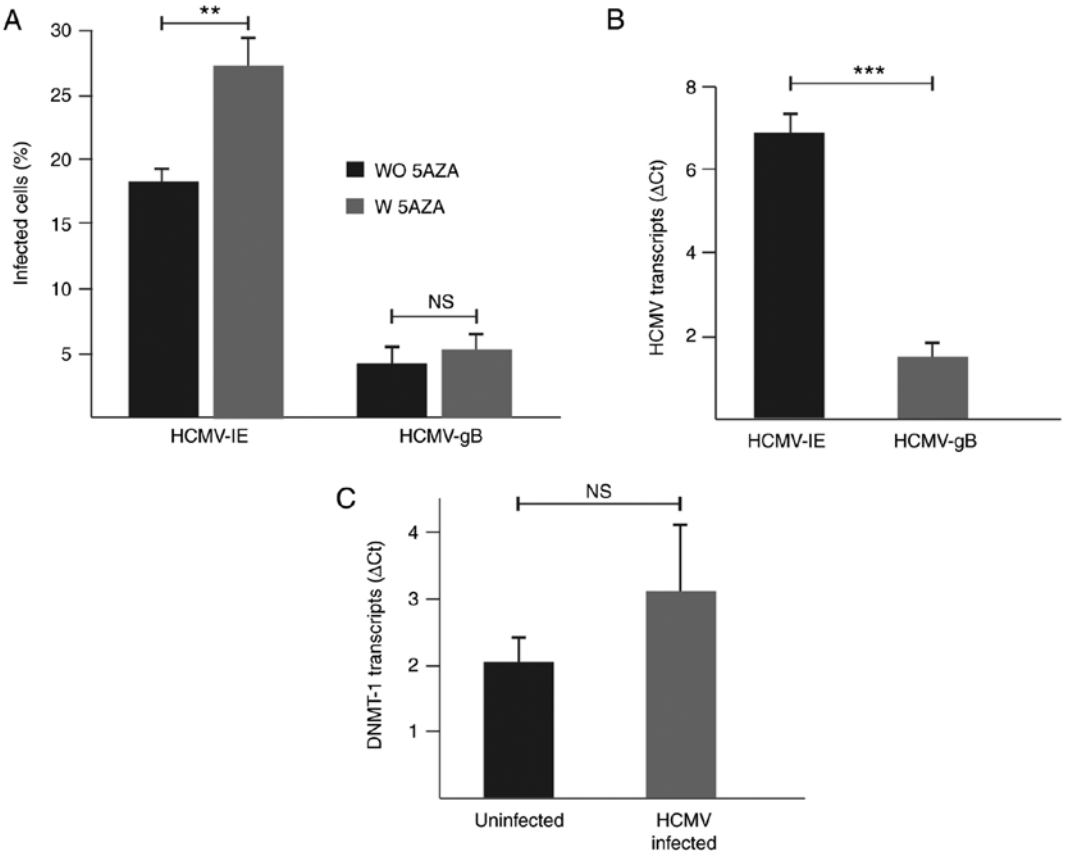

Figure 2. Expression of HCMV proteins and transcripts and in U343MG GBM cells. (A) Percentage of U343MG cells expressing HCMV-IE and HCMV-gB proteins in U343MG GBM cell group. (B) Detection of HCMV-IE and HCMV-gB transcripts at 3 days post-infection. (C) Unchanged levels of DNMT-1 transcripts in HCMV-infected cells compared with uninfected cells. **P<0.01, ***P<0.001. DNMT-1, DNA methyltransferase 1; HCMV, human cytomegalovirus; GBM, glioblastoma; gB, glycoprotein gB; IE, immediate early; NS, not significant.

5AZA treatment does not modulate transcription levels of viral genes. U343MG GBM cells were treated with 5AZA and subsequently infected with HCMV for 3 days. Untreated and/or uninfected cells were used as the controls. In 5AZA-treated HCMV-infected cells, HCMV-IE and HCMV-gB proteins were detected in 27 and 5% of the cells, respectively (P=0.002; Fig. 2A). No significant differences were observed in the levels of HCMV-IE and HCMV-gB viral transcripts, as well as DNMT-1 transcripts in 5AZA-treated HCMV-infected cells compared with untreated HCMV-infected cells (data not shown; HCMV-IE, P=0.2; HCMV-gB, P=0.4; DNMT-1, P=0.1).

Antiviral treatment prevents extranuclear localization of DNMT-1. Uninfected and HCMV-infected U343MG GBM cells were treated with the antiviral drug Ganciclovir® in order to inhibit the expression of late viral genes. As expected, HCMV-gB protein expression was not detected in the Ganciclovir®-treated cells (Fig. 1). DNMT-1 expression was retained in the nuclei of HCMV-infected antiviral treated cells (Fig. 1).

U343MG cell invasion is significantly decreased by HCMV infection. Untreated HCMV-infected U343MG cell invasion was significantly decreased compared with the untreated uninfected cells (P=0.03). Cell invasion was further decreased with 5AZA treatment in infected cells, compared with the untreated uninfected cells (P=0.0009). However, a borderline significant decrease in invasive ability was observed in treated infected cells compared with the untreated infected cells (P=0.05). A significant difference in invasion was observed between the 5AZA-treated uninfected and HCMV-infected cells (P=0.02; Fig. 3A).

5AZA treatment significantly decreases the proliferation of uninfected and HCMV-infected U343MG GBM cells. The proliferation of 5AZA-treated uninfected and HCMV-infected cells was significantly decreased compared with the untreated uninfected cells (P<0.0001) and untreated HCMV infected cells (P<0.0001), respectively (Fig. 3B). No significant differences in proliferation were detected between the untreated uninfected cells, compared with the untreated infected cells (P=0.4). The proliferation ability of 5AZA-treated uninfected cells was significantly decreased compared with the 5AZA-treated HCMV-infected cells (P=0.02). Furthermore, the proliferation of 5AZA-treated HCMV-infected cells was significantly decreased compare to untreated uninfected cells (P=0.0001; Fig. 3B).

Cytoplasmic expression of DNMT-1 in the cells of vessel walls within the GBM. Expression of HCMV-IE, HCMV-gB, HCMV-late and DNMT-1 proteins was examined in available GBM tissues sections obtained from 5 patients by IHC (Table II). While the expression of HCMV-gB and

Table II. MGMT methylation status and detection of HCMV-proteins and DNMT-1 in GBM tissues by immunohistochemical staining.

	HCMV			MGMT	DNMT-1	
Patient	IE	Late	gB	Methylation	Nucleus	Cytoplasmic
1	4+	2+	1+	ND	Tumor cells	Vessel cells
2	4+	2+	1+	Unmethylated	Tumor cells	Tumor and vessel walls
3	4+	1+	1+	Unmethylated	Tumor cells	Vessel walls
4	3+	2+	1+	Methylated	Tumor cells	Vessel walls
5	4+	2+	1+	Methylated	Tumor cells	Vessel walls

0, negative, 1+, 5-10% positive cells; 2+, 10-25% positive cells; 3+, 25-50%; 4+, 50-75% positive cells. HCMV, human cytomegalovirus; IE, immediate early; gB, glycoprotein-B; MGMT, methylated-DNA-protein-cysteine methyltransferase; ND, not determined.

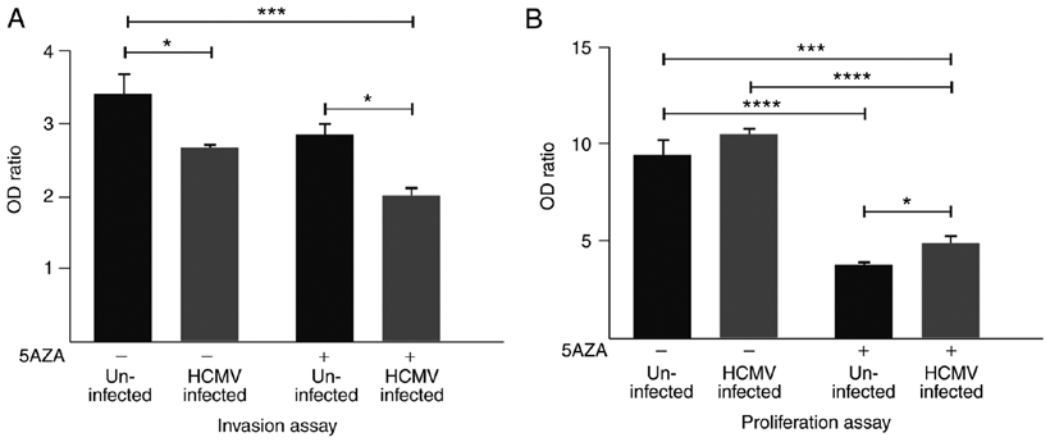

Figure 3. U343MG cell invasion is significantly decreased by HCMV infection. (A) Invasion assays were performed in U343MG GBM cells. Invasion was significantly decreased in 5AZA-treated HCMV-infected cells, compared with untreated or treated uninfected cells. (B) Proliferation assays were performed in U343MG GBM cells. Proliferation was significantly decreased in 5AZA-treated uninfected and HCMV-infected cells compared with untreated uninfected and infected cells, respectively. *P<0.05, ***P<0.001, ****P<0.0001. HCMV, human cytomegalovirus; GBM, glioblastoma; 5AZA, 5-azacytidine.

late proteins was low and rarely detected in GBM tissues, the expression of HCMV-IE was frequently detected at different levels in tumor cells and in the cells of blood vessel walls within the tumors. Furthermore, while DNMT-1 was expressed in the nuclei of tissue tumor cells, it was also present in the extranuclear space of cells in the vessel wall within the tumors (Fig. 4A and B depict two patients with primary GBM). In one GBM patient (patient number 2) who had deletion in chromosome 1p19q, DNMT-1 was expressed in the extranuclear space in the majority of tumor cells as well as in the vessel walls.

Discussion

HCMV latency has been unambiguously linked to epigenetic states by viral histone deacetylation, and viral reactivation to histone acetylation (9,11,12,15). We previously reported that HCMV replication increased in DNA methylation-inhibited non-tumor (human umbilical vein endothelial cells) and tumor cells (MB) (25). However, in the present study, 5AZA treatment of HCMV-infected GBM cells did not significantly affect viral replication, although the treatment appeared to increase the number of cells expressing HCMV-IE proteins by 10%. This effect is likely due to the decreased number of cells as a result of 5AZA treatment, as untreated and 5AZA-treated cells were infected with an equal MOI of virus. As DNMT-1 and viral DNA transcript levels were not significantly altered by HCMV infection in U343 cells, the western blot analysis was not included. The opposite results on HCMV replication upon 5AZA treatment in MB (25) and GBM tumors suggests that the different biology and epigenetic mechanisms involved may affect the oncogenic signaling pathways. This distinction may be highly important when considering epigenetic therapies of cancers that harbor HCMV, such as MB and GBM. Furthermore, as we have previously reported for non-cancer cells and MB cells (25), infection of GBM cells by HCMV causes DNMT-1 accumulation in the cytoplasmic/extranuclear space, rather than the nucleus. This was found to be associated with the presence of HCMV late protein(s), as demonstrated by IHC in the present study.

As DNMT-1 is likely to only function in the nucleus (28), the DNMT inhibitor 5AZA was used to investigate the effect of reduced DNMT-1 activity on the cell proliferation and invasion. Both uninfected and HCMV-infected cells had evidently reduced proliferation following 5AZA treatment. HCMV infection of U343MG cells alone did not alter cell

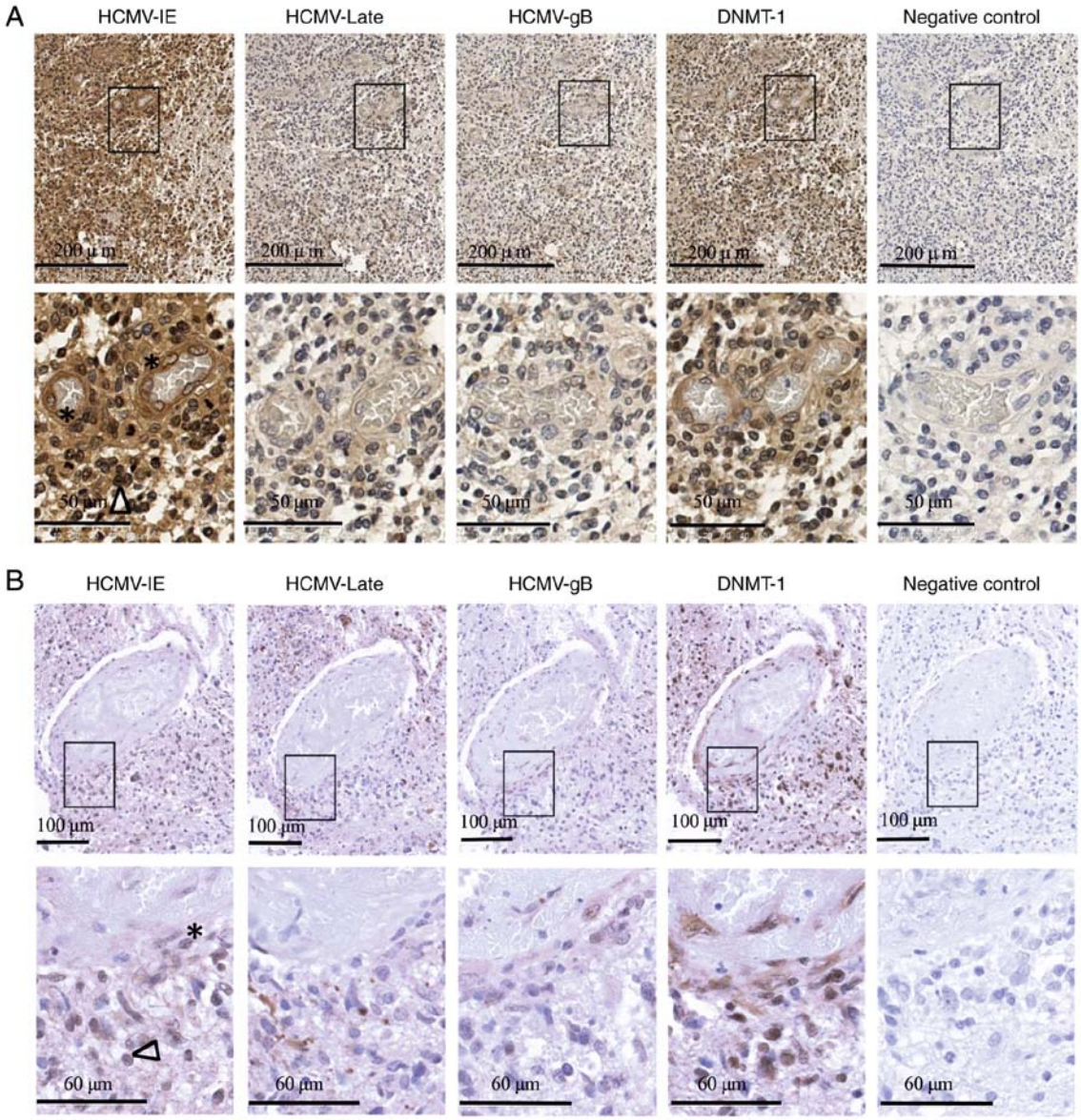

Figure 4. Detection of HCMV proteins in GBM tumor samples. (A and B) Expression of HCMV-IE, -late, -gB and DNMT-1 in cancer cells (Δ) and the cells of vessel walls (*) within the GBM tumor obtained from (A) primary GBM with chromosome 1p19q and (B) primary GBM patients. DNA methyltransferase 1; HCMV, human cytomegalovirus; GBM, glioblastoma; gB, glycoprotein gB; IE, immediate early.

proliferation, which has been previously reported in GBM cell lines (29). In contrast, the infection alone significantly decreased cell invasion, which was further reduced by 5AZA treatment. However, we have previously reported an increase in invasion by HCMV infection in U373MG cells (29). The differences in the invasion capacity of HCMV-infected GBM cells may be explained by different genetic background, global gene methylation status and infection rate, as cells were infected for 5 days in our previous study, compared to 3 days in the present study.

Whether or not the observed effects are related to DNMT relocalization or to other mechanisms requires further investigation. This information may be of importance when considering treatment of GBM patients with methylation inhibitors. However, since a majority of GBM tumors are likely to be infected by HCMV, a limitation of the present study is that there was no access HCMV negative GBM tissue sections. In our laboratory, the expression of various HCMV proteins in hundreds of GBM tissues have been detected, and only a limited number are HCMV protein negative. Therefore, HCMV-protein negative tissue specimens could not be examined for the current study.

We have previously reported extended overall survival in GBM patients receiving continued treatment with the antiviral drug Valcyte® as an add-on for routine treatment (30,31). Hypothetically, based on the findings of the present study, GBM patients may benefit from a combination therapy including epigenetic drugs and Valcyte® with the aim of reducing the proliferation and invasion of tumor cells. This must, however, be thoroughly investigated prior to clinical trial investigation. Notably, cytoplasmic localization of DNMT-1 was predominantly observed in vessel walls, with or without HCMV-gB or pan late protein expression within the GBM tumor tissues. Further, in one GBM patient who had

a co-deletion in chromosome 1p/19q, also known as oligodendroglioma, DNMT-1 was detected in the extranuclear space in the majority of tumor cells as well as in the vessel walls within the tumors. The contribution of the 1p/19q deletion in oligodendroglioma oncogenesis is still unknown. GBM patients with 1p/19q deletions are known to be chemosensitive with longer overall survival (OS) and progression free survival (PFS) regardless of the treatment they receive (32-36). Furthermore, the link between the 1p/19q co-deletion and epigenetic alterations in the course of demethylation/hypomethylation has previously been studied. A positive association between isocitrate dehydrogenase (IDH) promoter mutations and oncometabolite R-2-hydroxyglutarate (R-2-HG) production was reported (37,38). Various enzymes, including epigenetic regulatory methylcytosine dioxygenase TET (TET)2, are inhibited by R-2-HG, causing hypermethylation of DNA (37,38). However, in the present study, DNMT-1 was localized in the cytoplasm of many tumor cells and the cells of vessel walls within the tumor in the patient with 1p/19q co-deletion. Additionally, in this patient, the methylated-DNA-protein-cysteine methyltransferase (MGMT) promoter was unmethylated and tumor regrowth occurred at 8 months, resulting in an overall survival of only 14.5 months. Of note, HCMV-IE protein was frequently expressed at various levels both in the tumor cells and in the cells of vessel walls within the GBM tumors examined in this study. In an *in vivo* scenario in GBM tumors where HCMV-IE is frequently expressed while HCMV-late genes are rarely expressed, a direct or indirect effect of HCMV-IE expression on DNMT-1 localization, increased tumor cell proliferation, and invasion cannot be excluded. Hypothetically, the absence of nuclear and presence of extranuclear localization of DNMTs in HCMV-infected vessel cells in the GBM tissues may lead to demethylation/hypomethylation of DNA and subsequent alteration of cellular characteristics, as well as potential induction of factors that impact angiogenesis and tumorigenesis. Furthermore, exposure of the cells to inflammatory factors and cytokines such as prostaglandin E2 (PGE2), interleukin (IL)-1, IL-6 and IL-8, alters levels and/or activity of enzymes involved in DNA and histone methylation, resulting in global and potentially gene-specific changes which could in turn lead to changes in cellular phenotypes over time, resulting in increased invasion and proliferation (39,40). Furthermore, increased production of inflammatory factors and cytokines such as PGE2, IL-2, IL-6 and IL-8 in response to HCMV infection have previously been reported (41,42).

In the present study, the proliferation and invasion ability of uninfected and HCMV infected U343MG cells with or without 5AZA treatment was investigated. A significant decrease in the proliferation of both treated uninfected as well as infected cells was detected. Conversely, invasion capacity was significantly decreased both by infection alone, as well as by 5AZA treatment in uninfected and infected cells. Based on this observation, the potential benefits of this treatment in HCMV-infected GBM patients to inhibit tumor invasion and proliferation should be further investigated.

The importance of DNA methylation in GBM is suggested by previous studies, which have reported hypomethylation of oncogenes and hypermethylation of tumor suppressor genes in GBM (43-46). Nagarajan and Costello (44) reported hypomethylation in 76 promoters including TERT and tumor protein 73, with subsequent increased transcript expression, suggesting that alterations in the gene regulatory mechanisms in GBM lead to the expression of oncogenic proteins. Hypermethylated suppressor genes in GBM include RB transcriptional corepressor 1, epithelial membrane protein 3, Ras association domain family member 1 isoform A (RASSF1A), cadherin 1 (CDH1) and zinc finger MYND-type containing 10, cell cycle regulators [cyclin dependent kinase inhibitor 2 (CDKN2)A and CDKN2B], DNA repair genes (MGMT, MutL homolog 1), and genes involved in tumor invasion and apoptosis [death-associated protein kinase 1 (DAPK) and TIMP metallopeptidase inhibitor 3] (44,46-54). However, gene specific methylation analysis should be performed in order to clarify the effects of HCMV infection on hyper- or hypomethylation of these genes. We performed gene specific methylation analyses of CDKNA, RASSF1A, MGMT, DAPK and CDH1 with a multiplex methylation specific PCR (MMSP) (55) assay in untreated, 5AZA treated, uninfected, and HCMV infected U343 MG cells. It was shown that all examined genes under the different conditions were hypermethylated, excluding CDKNA and DAPK, which were hypomethylated under all different conditions (data not shown). Optimization of a more accurate quantification method of gene hypermethylation is required. It is likely that there are heterogeneities in the methylation for several of these genes, but the method used is not linear with the amount of methylation, and therefore very small amounts of specific methylation may be revealed as greater than they are. In the future, these experiments will be performed in further detail.

As previously mentioned, extranuclear localization of DNMT-1 was observed in the cells of vessel walls within the GBM tumor tissues, as well as in the tumor cells of GBM tissues examined from a patient with co-deletion on chromosome 1p/19q. This may lead to altered cellular functions by demethylation of certain genes, resulting in a more oncogenic phenotype of these cells. It is of note that temozolomide treatment (alkylating agent) is more effective in GBM patients with a hypermethylated (downregulated) MGMT gene, which codes for a DNA repair enzyme (56,57). In GBM patients, MGMT promoter methylation is used as a biomarker for clinical temozolomide therapy management. Furthermore, hypermethylation and mutations in IDH1/2 with functional consequences for TET enzymes (involved in the demethylation of histones and DNA) as well as glial differentiation and establishment of glioma, have been reported (58). Since hypermethylation and silencing of tumor suppressors are frequently observed in cancer (59,60), and due to the reversible nature of epigenetic mechanisms, these are potential therapeutic cancer targets (61).

In conclusion, it was demonstrated that HCMV-infected GBM cells relocalize their DNMT-1 from the nucleus to the extranuclear space, which coincided with viral late protein production. The treatment of GBM cells with 5AZA resulted in reduced cell proliferation. In addition, cell invasion was decreased by infection. *In vivo*, most GBM tumors exhibited cytoplasmic localization of DNMT-1, predominantly in blood vessel cells, whereas cancer cells retained DNMT-1 expression in the nuclei. These findings may be of importance in further investigations into using DNA methylation and viral inhibitors in GBM therapy.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

AR and TJE designed the study; AE, NL, BD and AR performed the experiments; GS provide clinical samples and data; AR and AE analyzed and generated figures; AR and TJE wrote the manuscript. MRP, IN and LFH performed methylation experiments. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethical approval and consent to participate

Ethical permission was approved by local ethical committee at Karolinska Institute, Sweden (Dnr. 2008/628-31).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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