Activation of Telomerase by Human Cytomegalovirus

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Background
The mechanism by which human cytomegalovirus (HCMV) stimulates oncogenesis is unclear. Because cellular immortalization and transformation require telomerase activation by expression of the telomerase reverse transcriptase (hTERT) gene, we examined the role of HCMV in telomerase activation.

Methods
Normal human diploid fibroblasts (HDFs) and human malignant glioma (MG) cell lines were infected with HCMV or transfected with expression vectors encoding HCMV immediate early (IE) antigen 72 or 86. hTERT expression and promoter activity and telomerase activity were evaluated using reverse transcription–polymerase chain reaction, a luciferase reporter assay, and a telomeric repeat amplification protocol, respectively. hTERT promoter occupancy by the transcription factor Sp1, IE antigens, and histone deacetylases (HDACs) was assessed by chromatin immunoprecipitation. hTERT and IE protein expression in human primary glioblastoma multiforme (GBM) was determined immunohistochemically. All statistical tests were two-sided.

Results
In telomerase and hTERT-negative HDFs, HCMV infection induced constitutive hTERT expression and telomerase activation. The hTERT promoter activity in HDFs and MG cell lines was statistically significantly enhanced by HCMV in a dose-dependent manner (mean luciferase activity [arbitrary units] in control HDFs and in HDFs infected with HCMV at multiplicities of infection [MOIs] of 0.1 = 6 and 521, respectively, difference = 515, 95% CI = 178 to 850; mean activity at MOI of 1 and 10 = 8828 and 59923, respectively; \( P < .001 \) comparing control with HCMV-infected cells at all MOIs). Ectopic expression of HCMV IE-72 protein also stimulated hTERT promoter activity in HDFs. HCMV-mediated transactivation of the hTERT gene was dependent on the presence of Sp1-binding sites in the hTERT promoter and was accompanied by increases in Sp1 binding, acetylation of histone H3, and a reduction in HDAC binding at the core promoter. In specimens of GBM, HCMV IE and hTERT proteins were colocalized in malignant cells and their levels paralleled each other.

Conclusions
HCMV activates telomerase in both HDFs and malignant cells. These findings begin to reveal a novel mechanism by which HCMV infection may be linked to or modulate oncogenesis through telomerase activation.


Telomeres consist of (TTAGGG) repeated sequences and associated proteins at human chromosome ends and progressively shorten with each round of cell division. When telomeres become critically short, cells undergo irreversible growth arrest (senescence) (1). Therefore, such cell division–mediated telomere attrition prevents unlimited cell proliferation. To escape this proliferation barrier, human cells must acquire the ability to stabilize their telomeres. In most cases, telomere stabilization is achieved through activation of telomerase, an RNA-dependent DNA polymerase that lengthens telomeric DNA (1). Compelling evidence has accumulated that telomerase activation is crucial for cellular immortalization and malignant transformation (1): The enzyme is silent in most normal human cells because of the tight transcriptional repression of its catalytic unit, telomerase reverse transcriptase (hTERT), whereas up to 90% of human cancers express hTERT and have telomerase activity (2). Inhibition of hTERT expression or telomerase activity causes telomere dysfunction and impairs the proliferation and survival of cancer cells, and these cells eventually lose their oncogenic potential (3). Moreover, expression of hTERT or telomerase protects against apoptosis of cancer cells and...
stimulates cell proliferation independently of its telomere-lengthening function (4–6). Thus, hTERT and telomerase are of great importance for sustained proliferation and survival of cancer cells.

Human cytomegalovirus (HCMV), a member of the β-herpesvirus family, is a widespread human pathogen that persists for the lifetime of its host after a primary infection. The HCMV genome encodes more than 200 proteins, and the expression of these proteins occurs in a program of three sequential stages—immediate early (IE), early (E), and late (L)—after infection. These proteins have multiple biological activities that interfere with physiological functions in infected cells (7).

HCMV has long been suspected of contributing to the development of human cancer (7–9). Cytomegalovirus (CMV) is a mutagen (10) and is capable of transforming certain types of mammalian cells (11–14), and the HCMV-encoded 72-kDa IE protein (IE-72) and chemokine receptor US28 enhance cell proliferation, thereby promoting cellular transformation or tumor progression (15,16). HCMV DNA and HCMV-encoded gene products are present in various kinds of primary tumors (15,17–21). Cobbs et al. (18) observed that almost all primary tumor specimens from patients with glioblastoma multiforme (GBM) expressed HCMV IE proteins, whereas adjacent normal cells did not. Moreover, we have found that the level of HCMV infection in GBM is associated with patient survival (A. Rahbar and C. Söderberg-Nauclér, unpublished observations, 2008). However, a causal relationship between HCMV infection and cancer has not been established, and mechanistic insights into HCMV-mediated oncogenesis are few (7–9). Because a number of oncogenic viruses have been shown to induce hTERT expression and activate telomerase, thereby contributing to cellular transformation (22), we explored the potential link between HCMV infection and hTERT expression and telomerase activation.

Materials and Methods

Cells, Cell Culture, HCMV Strains, and Viral Infection

Human diploid fibroblasts (HDFs) including fetal lung–derived HL411 and MRC5, and human malignant glioma (MG) cell lines U178, U251, U343, U373, and U563 (provided by Dr M. Nistér, Cancer Center Karolinska, Karolinska Institutet, Stockholm, Sweden) were maintained in RPMI-1640 medium (Life Technologies, Paisley, Scotland) supplemented with 10% fetal calf serum, 2 mM -glutamine, and antibiotics (100 U/mL penicillin and streptomycin) at 37°C in 5% CO₂/95% air. Cells grown to approximately 70% confluence were exposed to one of the following HCMV strains at different multiplicities of infection (MOIs): AD169 (VR-538; American Type Culture Collection, Manassas, VA), VR1814 (an endothelial cell–adapted clinical isolate, provided by G. Gerna, University of Pavia, Pavia, Italy), TB40 (provided by G. Jahn and C. Sinzger, University of Tübingen, Tübingen, Baden-Württemberg, Germany), or Towne (American Type Culture Collection). Control cells were either not exposed to the virus or were incubated with virus that had been inactivated by UV irradiation (with the Stratagene UV Stratalinker 1800 (Stratagene, La Jolla, CA) set to auto-cross-link [the device setting] starting at 1200 × 100 µl and gradually decreasing down to 0, for six times) (23).

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction

Total cellular RNA was isolated from HDFs and glioma cell lines with an RNeasy Mini Kit (Qiagen, Hilden, North Rhine-Westphalia, Germany). cDNA was synthesized with Oligo dT₁₅ primers using a SuperScript III First-Strand Synthesis System for reverse transcription–polymerase chain reaction (RT-PCR; Invitrogen Life Technologies, Carlsbad, CA). hTERT mRNA expression was analyzed by RT-PCR with primers specific for hTERT mRNA (accession no. AF015950): 5′-CGGAAAGTGTGCTCGAGCAAA-3′ and 5′-GGATGAAGCGGAGTCTGCGA-3′.

Assessment of Telomerase Activity

Telomerase activity was assayed by use of a TeloTAGGG Telomerase PCR ELISA kit (Roche Diagnostics Scandinavia AB, Stockholm, Sweden) and a TRAPEZE kit (Chemicon International, Temeacula, CA), as recommended by the manufacturers. Control and HCMV-infected HDFs were suspended in 3-[3-cholamidopropyl] dimethyl ammonio] propane sulfoic acid (CHAPS) lysis buffer (10 mM Tris–HCl [pH 7.5], 1 mM MgCl₂, 1 mM EGTA, 0.1 mM phenylmethylsulfonfyl fluoride, 5 mM P₃-mercaptoethanol, 0.5% CHAPS, 10% glycerol) for 20 minutes on ice and centrifuged at 20000g for 20 minutes at 4°C. The amount of protein in the supernatants was determined using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). For each assay of telomerase activity, 1 µg of protein was used, and 30 polymerase chain reaction (PCR) cycles were performed after the elongation reaction using telomerase primers. The PCR products were resolved by polyacrylamide gel electrophoresis and visualized by staining with SYBR Green I (Roche), or detected using an enzyme-linked immunosorbent assay color reaction.

From the Editors
Luciferase Activity Assay in HCMV-Infected Cells

To examine the effect of HCMV infection on hTERT promoter activity, HDFs and glioma cells cultured in 24-well plates (25,000 cells per well) were transfected with a luciferase reporter plasmid (pT181) that contains the core promoter sequence of the hTERT 5’ region fused to the gene encoding firefly luciferase, or variations of this plasmid that contained core promoters that lacked functional Sp1- (pT181-Sp1-) or c-MYC- (pT181-MYC-) binding motifs (24,25) by use of Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Next day, various amounts (0.1, 1.0, and 10 MOIs) of HCMV AD169 or VR1814 strain were added to the cultures. To test for stimulation of hTERT promoter activity by HCMV IE proteins, the cells were cotransfected with the p181 reporter plasmid and immediate early antigens 72 (IE-72) or 86 (IE-86) expression plasmids in which expression of the viral proteins was driven by a CMV promoter (provided by Dr J. Nelson, Oregon Health and Science University, Portland, OR). Luciferase activity in cell lysates was determined with a dual luciferase reporter assay system (Promega, Madison, WI) 48 hours after transfection. hTERT promoter–driven firefly luciferase activity was normalized either against cellular protein concentrations or thymidine kinase promoter–driven Renilla luciferase activity and expressed as arbitrary units.

Immunoprecipitation and Immunoblotting Assays

Total cellular proteins from noninfected control and HCMV-infected HDFs were extracted with RIPA lysis buffer (25 mM Tris–HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]). For immunoprecipitation, cellular proteins (200 µg in 500 µL) were precipitated with 10 µg of IgG1-kappa monoclonal antibody against HCMV immediate early antigen (IEA) (Argene, Varilhes, France). Ten micrograms of mouse IgG1-kappa (MOPC-21; Sigma-Aldrich, St Louis, MO) served as an isotype control in the immuno-precipitation experiments. The precipitated material, or 30 µg of proteins in whole lysates for Sp1 analyses, was subjected to SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were probed with specific antibodies against histone deacetylases (HDAC–1, diluted 1:1500, or HDAC–2, diluted 1:1500), Sp1 (diluted 1:350) (all from Santa Cruz Biotechnology, Santa Cruz, CA), or β-actin (diluted 1:10000) (Abcam Plc, Cambridge, UK), followed by incubation with secondary anti-mouse or anti-rabbit horseradish peroxidase–conjugated IgG (diluted 1:10000). Detection of bound antibodies was done by chemiluminescence reactions (ECL; Amersham, Little Chalfont, UK).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed as described previously with minor modifications (26). Cellular DNA was cross-linked by incubating noninfected and HCMV-infected HDFs in medium containing 1% (vol/vol) formaldehyde for 10 minutes at 37°C followed by sonication (2 × 12 seconds at 1/3 output with a sonifier 250 [Schmersal, Wuppertal, North Rhine-Westphalia, Germany]) to solubilize chromatin and obtain DNA fragments approximately 200–1000 base pairs in length. Antibodies against Sp1 (5 µg; Santa Cruz Biotechnology), IEA (5 µg; Argene), HDAC–1 (5 µg; Millipore, Temecula, CA), HDAC–2 (5 µg; Santa Cruz Biotechnology), and acetylated histone H3 (2 µg; Millipore) were used to precipitate DNA fragments (from 1 million cells) bound by the corresponding proteins in a total of 1.5 mL volume. As negative controls, rabbit IgG (5 µg) or mouse IgG1-kappa (5 µg) was added to sonicated cross-linked DNA. The protein–DNA complex was incubated with protein A–Sepharose beads (Millipore), centrifuged at 1500g for 1 minute, eluted with 1% SDS-containing Tris–EDTA (TE) buffer, and reverse cross-linked by incubating the samples at 65°C overnight. After proteinase K treatment (Sigma-Aldrich), the DNA was extracted with phenol–chloroform (Sigma-Aldrich) and precipitated with ethanol. The recovered DNA was suspended in TE buffer and amplified by PCR with the primer pairs specific for the hTERT proximal promoter: 5’-CCAGGCGGCTCCAGTGAGAT-3’ (forward) and 5’-GGCTTTCCACGTGCACGAGGA-3’ (reverse). To further control for nonspecific antibody binding, the 3’ region of GAPDH gene was PCR amplified in parallel using the following primers: 5’-AAAGGCGCTTCAATCTCTT-3’ (forward) and 5’-GGTGGTCCAGGGGTCTTACT-3’ (reverse).

Immunofluorescence

Noninfected control and HCMV-infected MRC5 cells were analyzed by immunofluorescence microscopy for hTERT and HCMV proteins 3 days after infection. The cells were first incubated with Fc receptor block reagent (30 minutes), followed by Cyto Q Background Buster (30 minutes; Innovex Biosciences, Richmond, CA) to block nonspecific binding and then for 30 minutes at room temperature with a rabbit anti-hTERT primary antibody (diluted 1:200; Rockland Immunochemicals, San Diego, CA) and a mouse anti-HCMV IEA antibody (1:350 dilution; Chemicon International). The cells were then incubated with Alexa Fluor–488–conjugated donkey anti-rabbit antibody (1:500 dilution) and Alexa Fluor–594–conjugated donkey anti-mouse antibody (1:500 dilution; both from Invitrogen, Molecular Probes). Nuclei were counterstained with diamidino-2-phenylindole (DAPI). Cell fluorescence was analyzed with a Leica TCS SP5 (Leica Microsystems, Wetzlar, Germany) microscope equipped with filters for the detection of DAPI, Alexa Fluor–488, and Alexa Fluor–594. Images were acquired at ×63 magnification using an immersion oil objective and the Leica Application Suite Advanced Fluorescence software, and mounted in Adobe Photoshop.

Patient Samples and Immunohistochemistry

Paraffin-embedded primary tumor specimens were obtained from 10 GBM patients who were admitted to the Karolinska University Hospital during 2005. Written informed consent was obtained from all patients, and this study was approved by the ethics committee at Karolinska Institutet. Tissue sections (6 µm thick) were stained for HCMV IEA by use of high-sensitivity staining protocols as previously described (22) and for hTERT protein. Briefly, the sections were deparaffinized in clean xylene and absolute ethanol, rehydrated in water, postfixed with 4% neutral buffered formalin, treated with pepsin (Biogenex, San Ramon, CA), and then incubated in citrate buffer (Biogenex). The sections were treated with 3% H2O2 (Sigma-Aldrich) to inactivate endogenous peroxidase, avidin/biotin blocking kit (DakoCytomation, Glostrup,
Denmark), FC receptor block (Innovex Biosciences) and background buster (Innovex Biosciences) to block Fc receptor and other nonspecific bindings. Then, primary antibodies against HCMV IEA (diluted 1:350; Chemicon International) and hTERT (diluted 1:200; Rockland Immunochemicals) were added and incubated overnight at 4°C. Antibodies against smooth muscle cell α-actin (mouse IgG2a diluted 1:350; Biogenex) and rabbit IgG (diluted 1:400; R&D Systems, Minneapolis, MN) served as isotype controls. The sections were finally incubated with biotinylated anti-mouse or anti-rabbit antibodies (Biogenex). Immobilized antibodies were visualized with streptavidin-conjugated horseradish peroxidase and diaminobenzidine (Innovex Biosciences).

### Statistical Analysis

The differences in the hTERT promoter activity between control cell and HCMV-infected or IE-72–transfected cells and in telomerase activity between AD169- and VR1814-infected cells were analyzed using Mann–Whitney U tests. All statistical tests were two-sided and computed using SigmaStat3.1 software (Systat Software, Inc., Richmond, CA). P values less than .05 were considered statistically significant.

### Results

hTERT Expression and Telomerase Activation in HCMV-Infected HDFs

To assess the role of HCMV in regulating hTERT and telomerase expression, we infected HDF MRC5 cells, which do not normally express hTERT or telomerase activity (27–30), with HCMV strain AD169 and measured hTERT mRNA expression using RT-PCR. hTERT mRNA was detected 24 hours after infection at MOI 1.0 and persisted throughout the 7-day (168 hours) experiment (Figure 1, A). The hTERT mRNA induction similarly occurred in HL411 cells infected with AD169 (data not shown). UV-irradiated HCMV, which cannot replicate, did not stimulate hTERT expression (data not shown), indicating that HCMV gene expression rather than soluble factors in the viral stock was responsible for the stimulation of hTERT mRNA expression. All of the other strains of HCMV tested, including TB40, Towne, and VR1814, induced substantial hTERT mRNA expression in MRC5 cells (Figure 1, B). Because AD169 and VR1814 had similar and intermediate effects on hTERT mRNA induction, they were chosen as representative strains for additional experiments.

hTERT protein was detectable by immunofluorescence microscopy in HCMV-infected MRC5 cells but not in control cells (Figure 1, C). Moreover, hTERT signals were only found in HCMV IEA–positive cells as shown by immunofluorescence (Figure 1, C). IEA staining was observed in nuclei, and hTERT signals were predominantly localized in the cytoplasm, consistent with results of earlier studies of hTERT protein distribution that used the same hTERT antibody (31,32). Telomerase activity was also detected only in HCMV-infected MRC5 and HL411 cells, and the level was dependent on the MOI (Figure 1, D and E, and data for HL411 cells not shown). Both AD169 and VR1814 induced telomerase activity at comparable levels (telomerase activity [absorbance]: AD169 [MOI 1.0], mean = 0.84; VR1814 [MOI 1.0], mean = 1.06, difference = 0.22, 95% CI = −0.565 to 0.125, P = .15, Mann–Whitney U test) (Figure 1, F), which was similar to the hTERT mRNA expression profile in MRC5 cells infected with AD169 and VR1814 (Figure 2, B).

Effects of HCMV Infection and Ectopic IE-72 Expression on hTERT Promoter Activation in HDFs

Next, we examined whether HCMV regulates hTERT expression at the level of transcription, and if so, which of its gene products elicits the effect. In MRC5 cells transfected with p181, a plasmid in which luciferase expression is under the control of the hTERT promoter (Figure 2, A), HCMV infection statistically significantly increased luciferase activity in a dose-dependent fashion compared with that in noninfected control cells (mean luciferase activity [arbitrary units], control vs IE-72 at 0.1 = 59 ± 923, difference = 59 ± 917, 95% CI = 178 to 850; HCMV at MOI of 1 = 8828, difference relative to control = 8822, 95% CI = 4621 to 13 021; HCMV at MOI of 0.1 = 6 vs 521, difference = 515, 95% CI = 47 551 to 72 282; P < .001 comparing control with HCMV-infected cells at all MOIs) (Figure 2, B). In control MRC5 cells, the hTERT promoter was largely inactive, consistent with the stringent repression of hTERT transcription in normal human fibroblasts (27–30).

Because HCMV IE-72 and -86 proteins are transcriptional regulators (9,16), we hypothesized that they would activate the hTERT promoter when transiently expressed in HDFs. We therefore cotransfected p181 and IE-72 or -86 expression vectors into MRC5 cells and measured luciferase activity. The luciferase activity driven by the hTERT promoter increased by a statistically significant extent only in the cells cotransfected with the IE-72 expression vector (mean luciferase activity in control cells and cells transfected with 0.15 and 0.30 µg IE-72 plasmid = 0.5, 6.1, and 12.8, respectively, difference = 5.6 [control vs 0.15 µg IE-72] and 12.3 [control vs 0.30 µg IE-72], 95% CI = 5.2 to 6.1 [control vs 0.15 µg IE-72] and 9.2 to 15.5 [control vs 0.30 µg IE-72]; P = .029 comparing control vs IE-72 at 0.15 or 0.30 µg; P = .886 comparing control vs IE-86) (Figure 2, C). Thus, IE-72 rather than IE-86 contributed to the activation of the hTERT promoter in MRC5 cells.

A Requirement for Sp1 in the HCMV-Mediated Transactivation of hTERT

The hTERT proximal promoter region contains five Sp1-binding motifs and two E-boxes (Figure 2, A), and the transcription factors Sp1 and c-MYC/Max network proteins play key roles in regulating hTERT transcription under different cellular conditions (24,25). To examine the role of these proteins in the transcriptional activation of the hTERT gene by HCMV, we measured luciferase activity in MRC5 cells that were first transfected with variants of the p181 luciferase reporter–bearing mutations in all five Sp1 motifs (p181-Sp1−) or both E-boxes (p181-MYC−) and then infected with HCMV. There were no significant differences in hTERT promoter activity between p181-MYC− and wt p181–transfected cells when exposed to HCMV (luciferase activity in cells transfected with wt p181 at
Figure 1. Induction of telomerase reverse transcriptase (hTERT) expression and activation of telomerase by HCMV infection in HDFs. A) Dose-dependent induction of hTERT mRNA by HCMV. Similar results were obtained in four independent experiments. MRC5 cells were infected with the HCMV strain AD169 (AD) at different MOIs, harvested at the indicated time points, and analyzed for hTERT mRNA by RT-PCR. B) hTERT mRNA expression induced by different strains of HCMV. HDFs were infected with HCMV strain AD169 (AD), TB40 (TB), Towne (To), or VR1814 (VR) at MOI of 1.0; harvested 72 hours after infection; and analyzed for hTERT mRNA expression by RT-PCR. C) Expression of hTERT and HCMV IEA in HCMV-infected HDFs. MRC5 cells were infected with HCMV strain VR1814 at a MOI of 1.0 and were analyzed 72 hours later for hTERT and IEA expression by immunofluorescence. The hTERT (green) and IEA (red) signals were evaluated with the use of a confocal microscope equipped with a CCD camera and an image capturing and processing system. Rabbit IgG and mouse IgG2a were included as isotype controls for hTERT and IEA antibodies, respectively. Data are shown for one of four independent experiments. D, E, and F) Telomerase activation in HCMV-infected HDFs. In eight independent experiments, MRC5 cells were infected with HCMV strain AD169 for the indicated time periods and MOIs, and telomerase activity was assessed by use of a telomeric repeat amplification protocol. The final amplification products were analyzed by SYBR green staining of 12% polyacrylamide gels (D) or by a TeloTAGGG Telomerase PCR ELISA kit (E). In the latter assay, the level of telomerase activity was expressed as absorbance in arbitrary units, and error bars correspond to 95% confidence intervals. F) A comparison of telomerase activity between AD169- and VR1814-infected MRC5 cells. HCMV = human cytomegalovirus; HDFs = human diploid fibroblasts; IEA = immediate early antigen; MOI = multiplicities of infection; RT-PCR = reverse transcription–polymerase chain reaction OD = optical densities.

MOI 1.0 vs p181-MYC at MOI 1.0 = 8828 vs 9888, difference = 1060, 95% CI = 3408 to 7125, P = .535 (Figure 2, B). HCMV-infected cells transfected with p181-Sp1 had statistically significantly less luciferase activity than HCMV-infected cells transfected with wt p181, suggesting that binding of Sp1 is necessary for hTERT promoter activation (luciferase activity in cells transfected with wt p181 at MOI 1.0 vs p181-Sp1 at MOI 1.0 = 8828 vs 1527, difference = 7301, 95% CI = 3408 to 11 192, P < .001) (Figure 2, B).

Because HCMV was previously shown to increase Sp1 expression in HDFs (33,34), we examined expression of Sp1 and its binding to the hTERT promoter region in HDFs infected with HCMV. Sp1 expression increased in HCMV-infected MRC5 cells in a dose-dependent manner, as shown by immunoblot analyses.
Figure 2. Effect of HCMV infection on hTERT promoter activation and chromatin structure in HDFs. A) Schematic illustration of the hTERT proximal promoter, showing the locations of E-boxes and Sp1-binding motifs relative to the translational start codon (ATG). The hTERT proximal promoter sequence was inserted into a luciferase vector (p181). The Sp1 and MYC mutant variants (p181-Sp1 and p181-MYC) were derived from the wild-type p181 by mutation of all five Sp1 motifs or both E-boxes. B) Sp1 motif-dependent stimulation of the hTERT promoter activity by HCMV. Three independent experiments were performed. MRC5 cells were transfected with the reporter constructs containing wild type (wt) hTERT promoter sequences, or E-box (MYC-) or Sp1 (Sp1-) mutant variants, and infected with AD169 at different MOIs. Luciferase activity was analyzed 48 hours after infection, and mean activities and 95% confidence intervals were calculated. hTERT promoter activity was normalized against cellular protein concentrations and expressed as arbitrary units (relative luciferase activity). Mann–Whitney U-tests were used for two-sided P-value calculation. C) IE-72 – mediated activation of the hTERT promoter in HDFs. MRC5 cells were cotransfected with p181 hTERT promoter reporter plasmid and either the IE-72 or -86 expression vector. Promoter activity (expressed as arbitrary units) was analyzed 48 hours after transfection and normalized to Renilla luciferase activity driven by the thymidine kinase promoter. Mean values were derived from three independent experiments (error bars correspond to 95% confidence intervals). D) Immunoblot analysis of Sp1 expression in HCMV-infected HDFs. Left panel: MRC5 cells were infected with HCMV strain AD169 (AD) or VR1814 (VR) at an MOI of 1.0, and Sp1 expression was assessed 72 hours later. - Control cells without HCMV infection. Right panel: Dose-dependent stimulation of Sp1 expression in VR1814-infected cells. Similar results were obtained in five independent experiments. E) HCMV-mediated Sp1 and IEA binding to the hTERT promoter and alterations in the hTERT chromatin. MRC5 cells were infected with HCMV strain VR1814 or left without infection as controls, and chromatin immunoprecipitation assay was performed to analyze the presence of Sp1, IEA, HDAC-1 and -2, and acetylated histone H3 (AcH3) at the hTERT core promoter region. Rabbit IgG and mouse IgG1-kappa were included as isotype controls. F) Physical interaction between IE proteins and HDAC-1 and -2 in HCMV-infected HDFs. MRC5 cells were infected with VR1814 at an MOI 1.0 for 48 hours, and the cellular proteins were precipitated with an antibody to HCMV IEA. Upper panel: Left: The material immunoprecipitated by IEA and control (IgG1k: mouse IgG1-kappa) antibodies was detected using the HDAC-1 antibody; Right: Immunoblot analysis of HDAC-1 in control (-) and VR1841-infected cells (VR). Total cellular protein (4 µg, or 2% of input) derived from the same lysates used for immunoprecipitation was resolved on sodium dodecyl sulfate–polyacrylamide gel, transferred to a membrane, and detected with the HDAC-1–specific antibody. Lower panel: Immunoprecipitation and immunoblot analysis of HDAC-2 in control and HCMV-infected MRC5 cells. The experiments were performed as described above for HDAC-1 immunoprecipitation and immunoblotting. Molecular markers for HDAC-1 and -2: Top and bottom: 76 and 52 kDa, respectively. Actin immunoblot analyses served as a loading control. Molecular markers for actin: Top and bottom: 52 and 38 kDa, respectively. Similar results were obtained in three independent experiments. HCMV = human cytomegalovirus; HDAC = histone deacetylase; HDFs = human diploid fibroblasts; IE = immediate early; IEA = immediate early antigen; MOI = multiplicities of infection.

HCMV-Induced Chromatin Remodeling at the hTERT Promoter Region in HDFs

Inhibition of histone deacetylation in normal HDFs leads to hTERT transactivation that requires Sp1 or E-boxes (35,36). As determined by ChIP, in HCMV-infected MRC5 cells, acetylation of histone H3 was increased and HDAC-1 and -2 levels were reduced (Figure 2, E), consistent with local remodeling or opening of chromatin and active transcription of the hTERT gene.

IEA Interaction With HDAC-1 and -2 and Association With the hTERT Promoter in HCMV-Infected HDFs

In some cell types, IEA interacts with HDAC-1, -2, and -3, and inhibits their deacetylation activities (37–39). As detected by immunoblotting, HDAC-1 protein expression was increased in the HCMV-infected cells, and immunoprecipitation results showed that IE proteins specifically associated with both HDAC-1 and -2.
hTERT Promoter Activation and hTERT Expression in HCMV-Infected MG Cell Lines

Because telomerase activation is a critical step in the immortalization and transformation of human cells (1), we asked whether HCMV regulates hTERT expression in human malignant cells. Furthermore, because GBMs often express HCMV IE proteins (18), we examined the effect of HCMV infection and ectopic expression of IE-72 and -86 on the hTERT promoter activity in MG cells. HCMV infection increased hTERT promoter activity in a dose-dependent manner; at an MOI of 10, the activity increased 5- to 200-fold compared with that in control cells, depending on the MG cell line (P = .029) (Figure 3, A). The statistically significant difference in hTERT promoter activation appeared to reflect, in part, the HCMV infection efficiency. More than 90% of U251 cells were resistant to HCMV infection (data not shown), and these cells exhibited the smallest increases in the hTERT promoter activity (ie, only a fivefold increase at an MOI of 10). In all other cell lines, in which more than 85% of the cells were infected, the increases in promoter activity were much greater at MOI 10 (13- to 200-fold) (Figure 3, A). Furthermore, HCMV infection stimulated hTERT mRNA expression in all MG cell lines examined, except U251 (Figure 3, C).

To assess the effect of transient overexpression of IE-72 and -86 on hTERT promoter activity in MG cells, we cotransfected U373 cells with the p181 reporter plasmid and the IE-72 or -86 expression vector. As in MRC5 cells, transfection of IE-72 increased hTERT promoter activity (up to fivefold) compared with that in control U373 cells transfected with an empty vector, whereas ectopic expression of IE-86 had no detectable effect (mean luciferase activity, control vs IE-72 at 0.15 µg: 38 vs 101, difference = 63, 95% CI = 22 to 105, P = .01; control vs IE-86 at 0.15 µg = 38 vs 37, difference = 1, 95% CI = −43 to 40; P = .933) (Figure 3, B).

HCMV IEA and hTERT Protein Expression in Primary GBM

Finally, we analyzed HCMV IEA and hTERT protein expression in primary tumor specimens from 10 patients with GBM by immunohistochemistry. IEA and hTERT protein levels varied from patient to patient and within individual specimens. However, in all 10 specimens, hTERT staining was weak or undetectable in tumor cells with low levels of IEA but strong in the cells containing higher amounts of IEA (Figure 4). We observed that hTERT protein was present in both the nuclear and the cytoplasmic compartments of tumor cells. hTERT signals (red) colocalized with IEA signals (brown), as shown by double staining (Figure 4, A, top panel).

Discussion

HCMV has been implicated in the pathogenesis of human malignancies, including GBM, and prostate and colorectal cancer (17–21). However, the mechanism by which HCMV contributes to neoplasia is not well understood. Although a causal relationship between
HCMV and cancer has not been established (7,8), HCMV or its gene products appear to act as mutagens in oncogenesis (10,40). Moreover, HCMV IE proteins interfere with p53 and Rb checkpoint pathways, and they promote MG cell proliferation by enhancing PI3K/AKT activities (16,41). The HCMV-encoded chemokine receptor US28 contributes to cellular transformation by increasing the expression of vascular endothelial growth factor and enhancing cell growth and cell cycle progression (15). In addition, HCMV infection triggers inflammation (7,23), which has been implicated in oncogenesis (7). In this study, we reveal a novel mechanism through which HCMV may be linked to or modulate oncogenesis by demonstrating that HCMV stimulates hTERT transcription, thereby activating telomerase, which is essential for the immortalization and transformation of human cells.

The hTERT gene is tightly repressed in most normal human cells. However, multiple mechanisms stimulate hTERT transcription and consequently activate telomerase during the oncogenic process. We found that UV irradiation abolished HCMV-mediated hTERT induction (data not shown), indicating that HCMV gene expression, rather than soluble factors in the viral stock, is required for the stimulatory effect. It has recently been shown that another β-herpesvirus, which causes Marek disease in chickens, carries sequences homologous to telomerase RNA and thereby activates telomerase by expressing this viral RNA in host T cells to promote malignant transformation of T cells (42). However, the HCMV genome contains no sequences homologous to hTERT based on the National Center for Biotechnology Information blast analysis (data not shown). Our observations suggest that HCMV induces hTERT transcription through at least two mechanisms: The first involves Sp1. As shown in ChIP assays, HCMV infection led to the recruitment of the transcription factor Sp1 to the hTERT promoter, and mutation of the Sp1-binding motifs at the hTERT promoter statistically significantly attenuated the increase in promoter activity due to HCMV infection. By contrast, hTERT promoter activation by HCMV infection was independent of functional E-boxes, even though c-MYC is essential for hTERT transcription in many other contexts (28,29). How Sp1 is recruited to the hTERT promoter in HCMV-infected cells is unclear. Both the increase in Sp1 expression and the IEA occupancy of the hTERT promoter that we observed likely participate in the Sp1 recruitment process. In support of a second mechanism, we observed that HCMV IEA interacted with HDAC-1 and -2 in HCMV-infected MRC5 cells, which may prevent HDACs from binding to the hTERT promoter, thereby increasing histone H3 acetylation and chromatin remodeling at the hTERT promoter site. The two mechanisms are likely to be functionally linked because Sp1 requires HDAC inhibition to mediate hTERT promoter activation and hTERT expression (35,36). Furthermore, an interaction between HCMV IE-72 and -86 proteins and Sp1 to activate target gene transcription has been demonstrated previously (33,34,43).

HCMV IE proteins have been found in primary glioma tissues (18). To address the effect of HCMV on hTERT expression and functional and clinical implications in cancer, we analyzed glioma cell lines and primary GBM tumors for IEA and hTERT expression. Unlike HDFs, the MG cell lines expressed different levels of hTERT. However, in four of five tested cell lines, HCMV infection increased hTERT promoter activity by a statistically significant extent and enhanced hTERT mRNA levels. Because the extent of elevated hTERT promoter activity did not strictly parallel that of the increase in hTERT mRNA, regulation of hTERT expression at the posttranscriptional level or through other unknown mechanisms may be involved. Moreover, the concomitant expression of HCMV IEA and hTERT protein in primary GBM tumors underscores the clinical relevance of HCMV-mediated activation of telomerase.

Telomerase activation is common to the process of transformation by a number of human tumor viruses (22), including Epstein–Barr virus, Kaposi sarcoma–associated herpesvirus,
human papillomavirus, hepatitis B and C virus, and human T-cell leukemia virus-1, and, as we have shown here, HCMV induces transcription of hTERT and activates telomerase through different mechanisms (22). Because hTERT or telomerase can extend cellular life span, stimulate proliferation, and protect against apoptosis (1), HCMV-mediated telomerase activation may enable the virus to persist and spread in host cells and to contribute to the formation and progression of cancer. Thus, inhibiting hTERT expression might eliminate HCMV from the host, and targeting HCMV may have therapeutic effects on HCMV-associated malignancies mediated by changes in hTERT and telomerase expression.

Our study has limitations. First, although transient overexpression of IE-72 contributed to hTERT promoter activation by directly binding to the promoter, the effect was much weaker than that of HCMV infection alone. This difference might be explained by the fact that the abundance of IE proteins may be lower in transfected vs infected cells. However, there may also be additional or unidentified HCMV gene products that are responsible for telomerase activation. Second, because HCMV-infected fibroblasts die from virus-mediated lysis, we were unable to determine the functional consequence of HCMV-mediated telomerase activation or define whether hTERT expression or telomerase activation extends the life span of infected cells. Elucidation of these issues will offer further insights into biological and clinical significance of telomerase activation by HCMV.

References


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