Human Cytomegalovirus Infection Alters PC3 Prostate Carcinoma Cell Adhesion to Endothelial Cells and Extracellular Matrix[1]

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Abstract

The genome and antigens of human cytomegalovirus (HCMV) are frequently found in prostatic carcinoma. However, whether this infection is causative or is an epiphenomenon is not clear. We therefore investigated the ability of HCMV to promote metastatic processes, defined by tumor cell adhesion to the endothelium and extracellular matrix proteins. Experiments were based on the human prostate tumor cell line PC3, either infected with the HCMV strain Hi (HCMV^{Hi}) or transfected with cDNA encoding the HCMV-specific immediate early protein IEA1 (UL123) or IEA2 (UL122). HCMV^{Hi} upregulated PC3 adhesion to the endothelium and to the extracellular matrix proteins collagen, laminin, and fibronectin. The process was accompanied by enhancement of β_1-integrin surface expression, elevated levels of integrin-linked kinase, and phosphorylation of focal adhesion kinase. IEA1 or IEA2 did not modulate PC3 adhesion or β_1-integrin expression. Based on this *in vitro* model, we postulate a direct association between HCMV infection and prostate tumor transmigration, which is not dependent on IEA proteins. Integrin overexpression, combined with the modulation of integrin-dependent signalling, seems to be, at least in part, responsible for a more invasive PC3^{Hi} tumor cell phenotype. Elevated levels of c-myc found in IEA1-transfected or IEA2-transfected PC3 cell populations might promote further carcinogenic processes through accelerated cell proliferation.

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Introduction

Human cytomegalovirus (HCMV) is a ubiquitous herpes virus characterized by lifelong persistent infection. The frequency of infection ranges from 50% to 90% in the adult population and varies with socioeconomic status and, to some extent, geographic location. HCMV causes severe complications in immunocompromised individuals. HCMV is also a leading cause of virus-associated birth defects, is associated with atherosclerosis and coronary restenosis, and has been suggested as a cofactor in the progression of HIV-1 infection.

Based on serological and molecular studies, it has also been assumed that HCMV might be involved in the development and etiology of human malignancies, including colon carcinoma, Kaposi's sarcoma, cervical carcinoma, prostate adenocarcinoma, and pediatric malignancies such as Wilm's tumor and neuroblastoma [1]. The detection of viral DNA, mRNA, and/or antigens in tumor tissues led to the hypothesis that HCMV catalyzes oncogenic processes (oncomodulation), which might result in a more malignant phenotype.

However, this hypothesis is controversial because HCMV is not restricted to tumorous organs but also infects organs in a high number of healthy individuals. Reports have also documented that viral DNA is often not retained in transformed cells and tumor samples [1]. Therefore, it is still uncertain whether HCMV contributes to oncomodulation and malignancy progression.

From a clinical viewpoint, the evaluation of HCMV's role in tumor pathology is highly desirable. Patients with HCMV$^+$ tumors might benefit from a combination of antiviral and antitumor therapy, if a link between HCMV infection and carcinogenesis can be proved. This is particularly true for patients suffering from prostate carcinoma—the most common cancer in men in the United States and in the western world, with increasing incidence and mortality rates [2,3]. Epidemiological studies indicate a significant association between prostate cancer incidence and increased exposure to sexually transmitted diseases, implying that a sexually transmissible agent(s) might increase the risk of prostate cancer [4,5]. Recent data

from Samanta et al. demonstrate that persistent infection by HCMV, which can be sexually transmitted, occurs in a high percentage of neoplastic prostatic epithelial lesions. Based on the specific detection of HCMV nucleic acids and proteins in biopsy specimens of prostate carcinoma patients, Samanta et al. [6] postulated a direct contribution of HCMV to the history of prostatic cancer. Nevertheless, the specific localization of virus material within a tumor is not a definitive evidence of a causal link between HCMV infection and human cancer.

In fact, the significance of HCMV in the pathogenesis of prostate cancer has remained obscure. Contrary to the abovementioned findings, polymerase chain reaction in prostatic tissue samples obtained during radical prosta- tectomy did not detect the genome sequences of HCMV in any sample, thus failing to support a viral etiology of pros- tate cancer [7].

To determine whether HCMV plays a role in prostate cancer or whether the virus is only a passenger in tumor cells, we examined the influence of HCMV on the adhesive capacity of tumor cells in a well-established cell culture model [8]. Experiments were based on the human prostate tumor cell line PC3, which was either infected with the HCMV strain Hi (HCMV^{Hi}) or transfected with cDNA encoding the HCMV-specific immediate early protein IEA1 (UL123) or IEA2 (UL122). Our data present strong evidence that HCMV upregulates prostate tumor cell adhesion to the endothelium and to the extracellular matrix proteins collagen, laminin, and fibronectin. HCMV^{Hi} infection leads to enhancement of β_1-integrin surface expression, elevated levels of integrin- linked kinase (ILK), and phosphorylation of focal adhesion kinase (FAK), as well as altered c-myc expression. This is the first report to reveal a direct link between HCMV infection and prostate carcinoma invasion.

Materials and Methods

Cell Cultures

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical veins and harvested by enzy- matic treatment with chymotrypsin. HUVEC were grown in Medium 199 (M199; Biozol, Munich, Germany) supple- mented with 10% fetal calf serum (FCS), 10% pooled human serum, 20 μg/ml endothelial cell growth factor (Boehringer Mannheim, Mannheim, Germany), 0.1% heparin, 100 ng/ml gentamicin, and 20 mM HEPES buffer (pH 7.4). Subcultures from passages 2 to 6 were selected for experimental use.

The human prostate tumor cell lines PC3, DU-145, and LNCaP were obtained from DSMZ (Braunschweig, Ger- many). Tumor cells were grown and subcultured in RPMI 1640 (Gibco/Invitrogen, Karlsruhe, Germany). The medium contained 10% FCS, 2% HEPES buffer (1 M, pH 7.4), 2% glutamine, and 1% penicillin/streptomycin. Subcultures from passages 7 to 11 were selected for experimental use. Cell viability was determined by trypan blue (Gibco/Invitrogen). All cells were maintained in an incubator at 37°C, with a 5% CO_2 humidified atmosphere.

HCMV Infection

HCMV^{Hi} was isolated from the bronchial lavage of an HIV patient. HCMV^{Hi} was cultured in human foreskin fibroblasts (HFF) and incubated in MEM (Biochrom, Berlin, Germany) supplemented with 2% FCS, 20 mM HEPES buffer, 2% bicarbonate, and 1% penicillin/streptomycin. Virus titer was determined by plaque titration in HFF, as described pre- viously [9]. PC3 cells were infected at a multiplicity of infection (MOI) of 0.1 or 1 in RPMI 1640 supplemented with 2% FCS (PC3^{Hi}). After 24 hours at 37°C, the medium was removed and replaced by RPMI 1640 supplemented with 10% FCS for 1 or 3 days. Immunoperoxidase staining against the HCMV-specific immediate early protein (72-kDa immediate early Ag, IEA, UL123; Biotrend, Köln, Germany) or the nuclear late protein (67-kDa late Ag, LA; DuPont, Bad Hom- burg, Germany) was carried out routinely after each sub- culture [10]. For control purposes, an irrelevant antibody directed against HSV glycoprotein B was used. The efficiency of HCMV^{Hi} infection was always about 30% related to IEA- expressing cells. Mock-infected inocula were prepared in an identical fashion, except that cell monolayers were not infected with HCMV^{Hi}. Virus inactivation was carried out by the exposure of virus solution to UV light (220 V, 12 W) for 15 minutes [11]. Samples of irradiated virus were then used to infect PC3. UV-irradiated samples were free of infectious virus, as detected by plaque titration.

Transfection Procedure

PC3 cells were transfected using the Effectene Trans- fection assay (Qiagen, Hilden, Germany). cDNA encoding HCMV IEA1 (UL123) was cloned into the pBS$^{+/-}$ vector (Stratagene, Heidelberg, Germany) and inserted in the expression vector pHM135. cDNA encoding HCMV IEA2 (UL122) was cloned into the pBS$^{+/-}$ vector (Stratagene) and inserted in the expression vector pHM134. cDNA encod- ing HCMV IEA1 and IEA2 was cloned into the pUC18 vector (Fermentas, St. Leon-Rot, Germany) and inserted in the expression vector pHM127 (expression vectors were a kind gift from T. Stamminger, University of Erlangen-Nürnberg, Erlangen, Germany). Control cells were transfected with the vectors alone. Subconfluent PC3 cells were transfected with 8 g of DNA for 6 hours at 37°C in 75-cm^2 culture flasks. Sub- sequently, cells were washed with phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} and were incubated overnight in a complete medium. The efficiency of transfection was investigated by immunoperoxidase staining against the HCMV-specific immediate early protein. The average rate of transfected PC3 cells was around 45% (PC3^{pHM135}, PC3^{pHM134}, and PC3^{pHM127}). The viability of the cells, which was controlled by trypan blue dye exclusion, was > 90%.

Tumor Cell Adhesion to HUVEC

HUVEC were transferred to six-well multiplates (Falcon Primaria; BD Biosciences, Heidelberg, Germany) in complete HUVEC medium. When confluency was reached, PC3 cells were detached from culture flasks by accutase treatment (PAA Laboratories, Cölbe, Germany), and 0.5 × 10^6 cells were then added to the HUVEC monolayer for 60 minutes.

Subsequently, nonadherent tumor cells were washed off using warmed (37°C) Medium 199. The remaining cells were fixed with 1% glutaraldehyde. In each experimental setting (PC3 vs PC3^{Hi}), adherent tumor cells were counted in five different fields of a defined size (5 × 0.25 mm^2) using a phase-contrast microscope, and the mean cellular adhesion rate was calculated.

Attachment of Tumor Cells to Extracellular Matrix Components

Twenty-four-well plates were coated overnight with collagen (diluted to 100 μg/ml in PBS; Seromed Biochrom, Berlin, Germany), laminin (diluted to 50 μg/ml in PBS; BD Biosciences), or fibronectin (diluted to 50 μg/ml in PBS; BD Biosciences). Plastic dishes served as background control. Plates were washed with 1% bovine serum albumin (BSA) in PBS to block nonspecific cell adhesion. Thereafter, 1×10^5 tumor cells/well were added for 60 minutes. Subsequently, nonadherent tumor cells were washed off, and the remaining adherent cells were fixed with 1% glutaraldehyde and counted microscopically. The mean cellular adhesion rate (adherent cells$_{coated\ well}$ − adherent cells$_{background}$) was calculated from five different observation fields.

FACScan Analysis

The oncoprotein expression, as well as the integrin expression, of PC3 vs PC3^{Hi} tumor cells was investigated by flow cytometry. To determine if protein/integrin modulation was restricted to HCMV-infected PC3 cells, cell cultures were double-stained using monoclonal antibodies directed against the HCMV-specific 72-kDa IEA and against the protein in question. Tumor cells were harvested by accutase treatment and washed in blocking solution (PBS and 0.5% BSA). To carry out integrin surface analysis, cells were fixed with 100 μl of fixation medium (Fix&Perm; Biozol-An der Grub Bioresearch, Eching, Germany) and washed twice in blocking solution (PBS and 0.5% BSA). Subsequently, they were incubated for 60 minutes at 4°C with 100 μl of permeabilization medium (Fix&Perm) together with the monoclonal antibody anti−72-kDa IEA (1:50, mouse IgG1-K; Biotrend). This process was repeated to allow labeling with the fluorescein isothiocyanate (FITC)−conjugated anti−IgK monoclonal antibody (1:20, rat−antimouse IgK; Becton Dickinson, Heidelberg, Germany). In a further step, tumor cells were marked with the PE-conjugated monoclonal antibodies anti-CD49a ($\alpha_1\beta_1$), anti-CD49b ($\alpha_2\beta_1$), anti-CD49c ($\alpha_3\beta_1$), anti-CD49d ($\alpha_4\beta_1$), anti-CD49e ($\alpha_5\beta_1$), or anti-CD49f ($\alpha_6\beta_1$; all from Becton Dickinson). Dot-plot quadrant analyses have been carried out to display the percentage distribution of PC3-expressing FITC−IEA and/or PE−integrin (IEA$^+$/integrin$^+$, IEA$^+$/integrin$^-$, IEA$^-$/integrin$^+$, and IEA$^-$/integrin$^-$). IEA$^-$ and IEA$^+$ cells were gated to obtain two distinct cell populations: population I (IEA$^+$) as HCMV-infected cells, and population II (IEA$^-$) as noninfected tumor cells. Integrin expression of both PC3 subtypes was then detected by FACScan analysis [FL-2H(log) channel histogram analysis; 1×10^4 cells/scan]. To evaluate the background staining of FITC-labeled IEA, FITC-conjugated anti-IgK (1:20, rat−antimouse) was used. Mouse IgG1−PE was used as an isotype control for integrin mouse IgG1−FITC−conjugated antibodies.

To analyze c-myc oncoproteins, cells were fixed with 5 ml of cold (−20°C) methanol/acetone (1:1, vol/vol) for 15 minutes. They were then incubated with the monoclonal antibody anti−72-kDa IEA and labeled with FITC, as described. Subsequently, monoclonal antibodies against c-myc (1:100, clone 9E10, mouse IgG1; Becton Dickinson) were added for 60 minutes, followed by an additional incubation with goat−antimouse IgG−PE (1:50; Becton Dickinson) for a further 30 to 60 minutes. PE-labeled IgG1 (1:50, goat−antimouse; Becton Dickinson) was used as the respective isotype control.

Western Blot Analysis

Total oncoprotein/integrin content in PC3 vs PC3^{Hi} cells was evaluated by Western blot analysis. Cells were lysed in lysis buffer containing 96.4% Triton X-100, 1% orthovanadate (2 mM), 1% okadic acid (10 mM), 1.2% PIM (12 μl/ml), and 0.4% PMSF (4 μl/ml). Proteins (50 μg) were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis on 10% gels for 60 minutes at 100 V, and then transferred to nitrocellulose membranes. After blocking, the membranes were incubated overnight with antibodies against CD49b (1:250, mouse IgG2a), CD49c (1:1000, rabbit), CD49d (1:200, mouse IgG), c-myc (1:250, mouse IgG1), ILK (clone 3), FAK (clone 77), and phospho-specific FAK (pY397, clone 18; all from Becton Dickinson). HRP-conjugated goat−antimouse or goat−antirabbit IgG (1:5000; Upstate Biotechnology, Lake Placid, NY) served as secondary antibodies. The membrane was briefly incubated with ECL detection reagent (Amersham-GE Healthcare, Braunschweig, Germany) to visualize the proteins and was exposed to an X-ray film (Hyperfilm EC; Amersham). β-Actin (1:1000, mouse; Sigma, Taufkirchen, Germany) served as internal control.

Reverse Transcriptase−Polymerase Chain Reaction (RT-PCR)

CD49d ($\alpha_4\beta_1$) mRNA was evaluated by RT-PCR. Tumor cells were seeded in 50-ml culture flasks (Falcon Primaria; 25-cm^2 growth area). Total RNA was isolated and extracted using QIAshredder (250) and RNeasy kit (Qiagen) according to the manufacturer's instruction. RNA samples were then treated with 80 U/ml RNase-free DNase I (Boehringer Mannheim) for 60 minutes at 37°C to eliminate amplifiable contaminating genomic DNA. Subsequently, samples were incubated for 10 minutes at 65°C to inactivate DNase. Complementary DNA was synthesized from 1 μg of total RNA per sample with a 60-minute incubation at 42°C, using the Moloney murine leukemia virus RT (Invitrogen, Karlsruhe, Germany) and oligo(dT) priming (Boehringer Mannheim). Amplification was carried out by gene-specific primers and Platinum-Taq polymerase (Invitrogen) in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). For DNA elimination, samples were treated with RNase-free DNase (Qiagen). cDNA synthesis (RT-PCR) was performed according to PowerScript protocol (Becton Dickinson). CD49d primer sequences were 5′-TGG CGT GGT ACA ACT TGA CTG-3′ and 5′-CAT GCG CAA CAT TCT CAT CCT-3′.

PC3 - HCMV^Hi
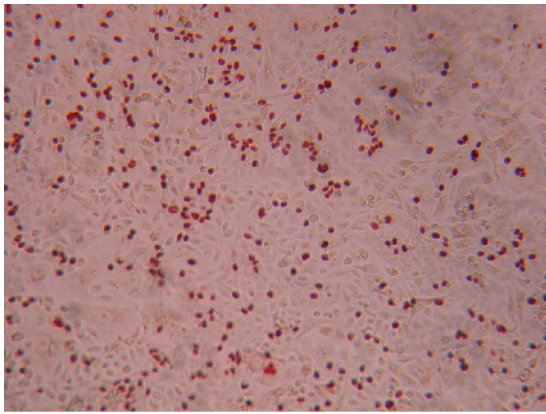
Figure 1. *Immunoperoxidase staining of HCMV (HCMV^Hi)–infected PC3 prostate tumor cells. PC3 cells were infected at an MOI of 1 and were stained against the HCMV-specific 72-kDa immediate early protein UL123. The efficiency of HCMV^Hi infection was about 30% (original magnification, ×100).*

Internal controls for PCR were performed by running parallel reaction mixtures with the housekeeping gene *GAPDH* (5′-ATC TTC CAG GAG CGA GAT CC-3′ and 5′-ACC ACT GAC ACG TTG GCA GT-3′). Reactions were performed in the presence of 0.5 μl of cDNA, with an initial incubation step at 95°C for 2 minutes. Cycling conditions consisted of denaturation at 95°C for 30 seconds, annealing at 30°C (GAPDH at 58°C) for 30 seconds, and extension at 72°C for 30 seconds over a total of 35 cycles. The reactions were completed by another incubation step at 72°C for 10 minutes. PCR products were subjected to electrophoresis in 1.5% agarose gel and visualized by ethidium bromide.

Statistical Analysis

All experiments were performed three to seven times. Statistical significance was investigated by Wilcoxon-Mann-Whitney *U* test. Differences were considered statistically significant at *P* < .05.

Results

HCMV^Hi Infection

In initial experiments, three different prostate tumor cells lines were exposed to HCMV^Hi. The infection rate for DU145 cells was < 1%, as evidenced by immunoperoxidase staining against the HCMV-specific immediate early protein UL123. The infection rate for LNCaP was 5%, whereas 30% of PC3 cells stained positively when infected at an MOI of 0.1 or 1 (Figure 1). Early 72-kDa proteins were produced in HCMV-infected PC3 cells within 24 hours postinfection (p.i.), whereas 67-kDa late proteins were produced within 72 hours p.i.

Adhesion of PC3^Hi to HUVEC

The binding of PC3 to HUVEC monolayers has been evaluated because adhesive interactions between tumor cells and the vessel wall reflect the first step of a hematogenous invasion cascade. Figure 2 shows PC3 tumor cell adhesion characteristics evaluated 1 and 3 days p.i. HCMV

infection led to a considerable enhancement of tumor cells, which adhered to HUVEC. An MOI of 1, rather than an MOI of 0.1, evoked stronger effects on PC3. The adhesion rate of tumor cells plated 1 day p.i. increased by 160.0 ± 39.9% (MOI = 1) *vs* 86.7 ± 46.2% (MOI = 0.1, mean ± SD, *n* = 7 experiments), compared to noninfected PC3 control cells. The adhesion rate of cells plated 3 days p.i. differed by 110.8 ± 30.5% (MOI = 1) *vs* 75.7 ± 13.2% (MOI = 0.1, mean ± SD, *n* = 7 experiments), compared to controls. Surprisingly, the treatment of PC3 with inactivated virus particles also induced a significant increase in tumor cell adhesion, which was most prominent 3 days p.i. (70.3 ± 22.7%, *n* = 7). Immunoperoxidase staining against the HCMV-specific IEA1 was negative in PC3 cells treated with inactivated viruses, excluding any contamination by intact virus material.

Adhesion of PC3^Hi to Collagen, Laminin, or Fibronectin Matrix

Once tumor cells have attached to the endothelium, direct interactions with underlying extracellular matrix structures
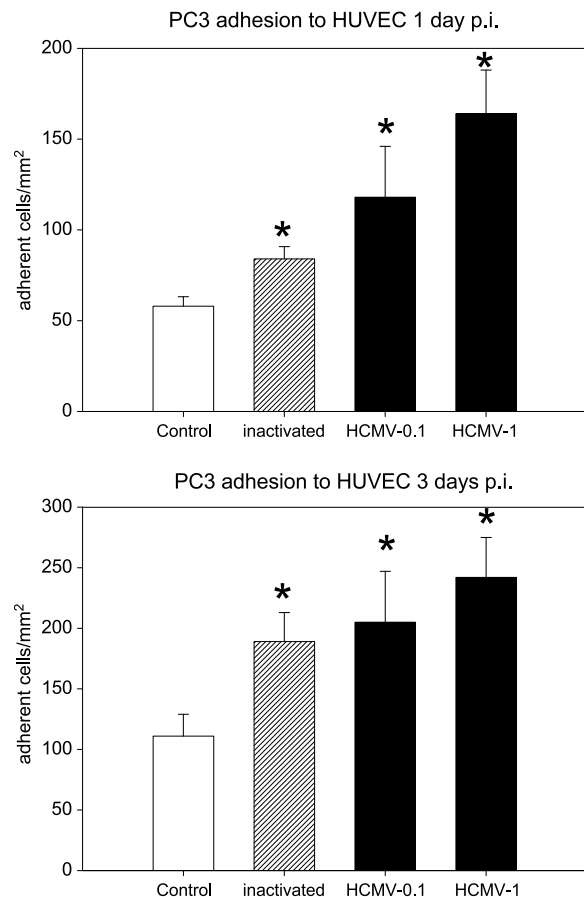
Figure 2. *HCMV promotes tumor cell adhesion. Mock-infected controls, HCMV^Hi-infected cells (MOI = 1 or 0.1), and UV-inactivated PC3 cells were cultured on HUVEC monolayers for 60 minutes. Nonadherent tumor cells were washed off in each sample, and the remaining cells were fixed and counted in five different fields (5 × 0.25 mm²) using a phase-contrast microscope. The upper figure presents PC3^Hi, infected for 24 hours; the lower figure is related to PC3^Hi, infected for 72 hours. Adhesion capacity is depicted as tumor cell adhesion per square millimeter (mean ± SD; one of seven representative experiments).*
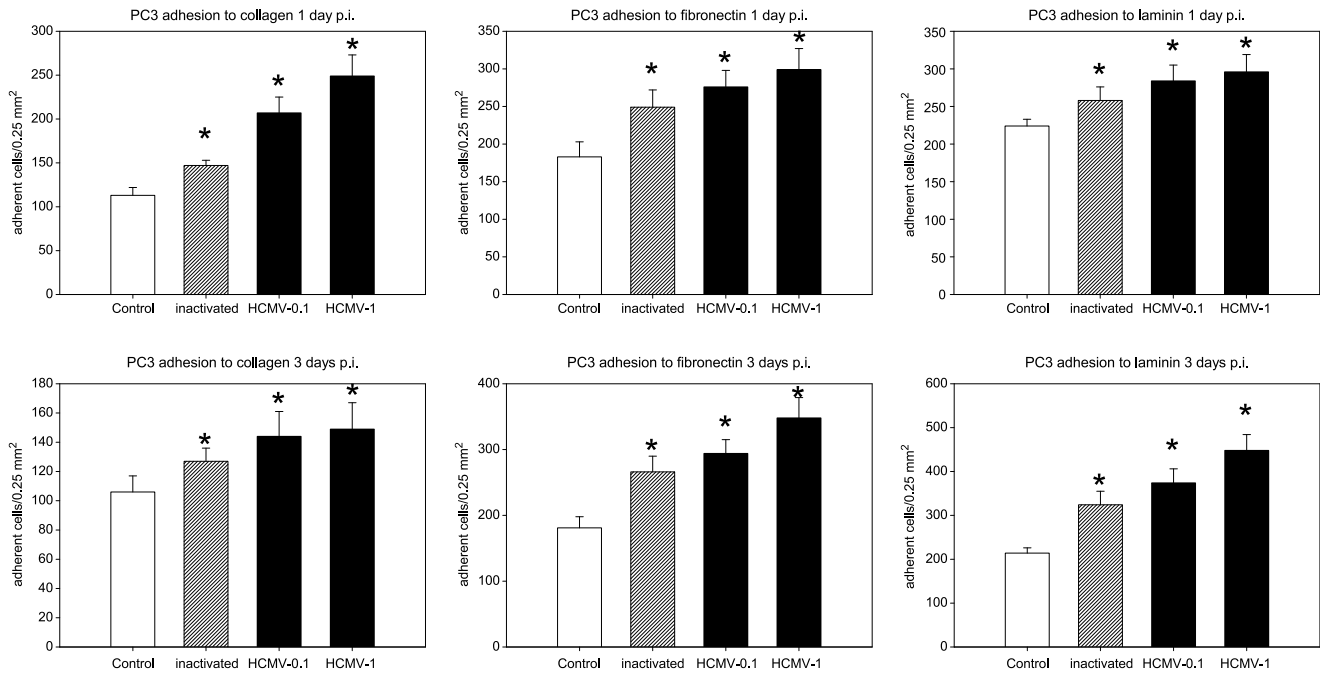
Figure 3. *The adhesion of prostate tumor cells to extracellular matrix proteins depends on HCMV^Hi. PC3 cells were added to immobilized fibronectin, laminin, or collagen for 60 minutes. Mock-infected controls, as well as HCMV^Hi-infected (MOI = 1 or 0.1) or UV-inactivated PC3 cells, were used. Nonadherent tumor cells were washed off in each sample, and the remaining cells were fixed and counted in five different fields (5 × 0.25 mm²) using a phase-contrast microscope. Mean values were calculated from five counts. Specific adhesion capacity (background adhesion on the plastic surface was subtracted from adhesion to matrix proteins) is depicted as counted cells per 0.25 mm². One of six representative experiments is shown.*

occur to allow subsequent transendothelial invasion. To further explore the functional significance of HCMV^Hi infection on the invasive behavior of tumor cells, PC3 cells were therefore added to extracellular matrix proteins, and the binding rate of infected *versus* noninfected cells was calculated. The number of adherent control cells differed with respect to the matrix protein used. Maximum adhesion capacity was measured on fibronectin-coated and laminin-coated plates; a lower binding rate was seen when culture plates were precoated with collagen (Figure 3). Tumor cells that had been inoculated with HCMV^Hi showed a binding activity significantly higher than that of control tumor cells. Similar to HUVEC adhesion studies, UV-inactivated viruses also triggered enhanced tumor cell binding. The effect was independent of the matrix component used and became evident 1 and 3 days p.i.

## HCMV^Hi Infection Leads to Enhancement of Integrin Surface Expression

Our data indicated that the adhesion of prostate tumor cells to the endothelium or matrix is altered by HCMV^Hi. We next explored whether virus infection leads to adhesion receptor alteration of the β_1-integrin family, whereby all subsequent experiments were related to an MOI of 1. Integrin β_1 subtypes are predominantly involved in cell adhesion to the extracellular matrix. α_1, α_2, and α_3 β_1 integrins can bind various matrix types, whereas α_4 and α_5 primarily react with fibronectin and α_6 primarily reacts with laminin. To distinguish PC3^Hi from PC3 in the same cell population, cell cultures were double-stained, and IEA^+ *versus* IEA^- tumor cells were

analyzed separately. Analysis of HCMV^Hi-infected tumor cell cultures demonstrated 28.4 ± 6.9% IEA^+ cells (mean from six experiments), the integrins of which were significantly elevated compared to the subset of noninfected IEA^- cells (3 days p.i.; Figure 4). Most prominently, α_4 integrin subtype was not expressed on IEA^- cells, but strong fluorescence signals were detected on IEA^+ cells. This might speak for the *de novo* synthesis of α_4 proteins; indeed, RT-PCR demonstrated only moderate α_4 mRNA activity in noninfected PC3 cells, whereas strong α_4 mRNA activity in PC3^Hi cells was noted. Furthermore, α_4 protein content increased in PC3 cells after HCMV^Hi infection (Figure 5). mRNA analysis of integrin β_{1A} did not reveal significant differences between HCMV-infected and control cells, which underlines flow cytometry data.

In strong contrast to HCMV^Hi-induced alterations of the β_1-integrin profile, UV-inactivated viruses exerted no quantitative changes on β_1 integrins, compared to controls (data not shown).

FAK (total and phosphorylated) and ILK were analyzed in ongoing experiments as surrogates for integrin-mediated signalling. In good accordance with integrin data, HCMV^Hi cells were characterized by enhanced ILK and FAKphospho levels (24 hours p.i.), whereas this effect was not seen in UV-inactivated or mock-infected cells (Figure 6).

The relevance of β_1 integrins for adhesion events was explored by blocking studies using β_1 monoclonal antibodies (clone 6S6). In this context, the adhesion of PC3 cells to HUVEC, fibronectin, laminin, or collagen was drastically inhibited by β_1-blocking antibodies (but not by corresponding
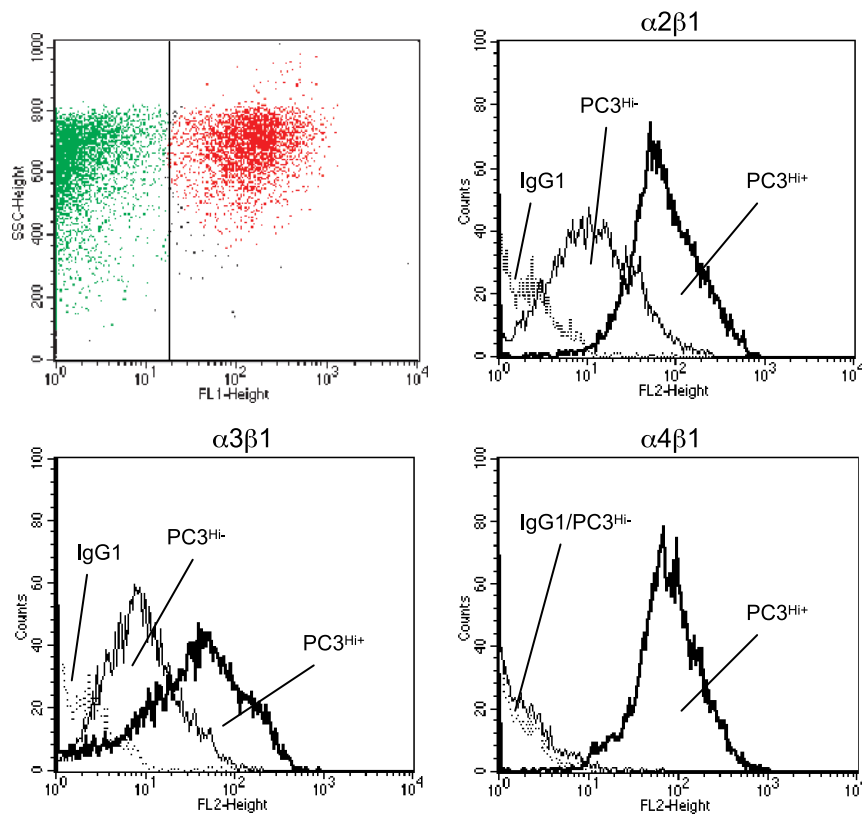
Figure 4. β_1-Integrin surface expression on $HCMV^{Hi}$-infected versus noninfected PC3 cells. To determine whether integrin modulation was restricted to $HCMV^{Hi}$-infected PC3 cells, cell cultures were double-stained using, on the first step, a monoclonal antibody directed against the HCMV-specific 72-kDa IEA, labeled with FITC. On the second step, PC3 cells were marked with the PE-conjugated monoclonal antibodies anti-CD49b ($\alpha_2\beta_1$), anti-CD49c ($\alpha_3\beta_1$), or anti-CD49d ($\alpha_4\beta_1$). IEA^- ($PC3^{Hi-}$) and IEA^+ cells ($PC3^{Hi+}$) were gated to obtain two distinct cell populations: population I (IEA^+) as HCMV-infected cells, and population II (IEA^-) as noninfected tumor cells. The integrin expression of both PC3 subtypes was then detected by FACScan analysis [FL-2H(log) channel histogram analysis; 1×10^4 cells/scan]. To evaluate background staining, mouse $IgG1-PE$ was used as isotype control.

IgG isotype controls), which speaks for a link between integrin upregulation and the enhancement of tumor cell−endothelium or tumor cell−matrix interactions, respectively (Figure 7).

$HCMV^{Hi}$ Modulates c-myc Expression

To further evaluate the oncomodulatory potential of $HCMV^{Hi}$, c-myc protein was analyzed subsequently. The flow cytometry of IEA^+ versus IEA^- cells in infected tumor cell cultures revealed only little intracellular c-myc protein in IEA^- cell populations, but significantly enhanced protein levels in IEA^+ cell populations. This was already detectable 1 day p.i., but became most evident 3 days p.i. (Figure 8). Flow cytometry data were confirmed by Western blot analysis, demonstrating increased c-myc in $PC3^{Hi}$ cells, compared to PC3 controls (Figure 8). The application of UV-inactivated viruses did not lead to any changes in c-myc (data not shown).

IEA1 and IEA2 Proteins Are Not Involved in the Regulation of Adhesion Processes

To investigate the role of immediate early and late proteins in regulating tumor cell adhesion, PC3 cells were transfected with cDNA encoding HCMV IEA1 (pHM135), HCMV IEA2 (pHM134), or both (pHM127). Interestingly, none of the transfected cell populations showed different adhesion character-
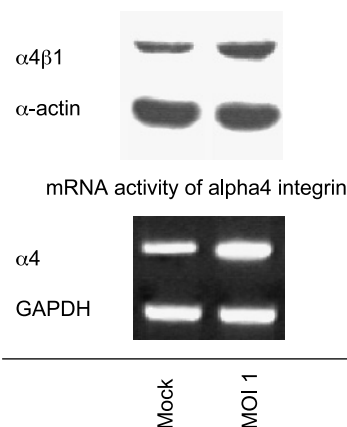
Figure 5. Top: Western blot analysis of $\alpha_4\beta_1$ integrin from the proteins of $HCMV^{Hi}$ versus mock-infected PC3 cells. Cell lysates were subjected to SDS-PAGE and blotted on the membrane incubated with anti$-\alpha_4\beta_1$ monoclonal antibody. β-Actin served as internal control. The figure shows one of three representative experiments. Bottom: RT-PCR analysis of $\alpha_4\beta_1$ integrin coding mRNA. $HCMV^{Hi}$ versus mock-infected PC3 cells were used. RNA was extracted, reverse-transcribed, and submitted to semiquantitative RT-PCR using gene-specific primers, as indicated in the Materials and Methods section. The figure shows one of three representative experiments.
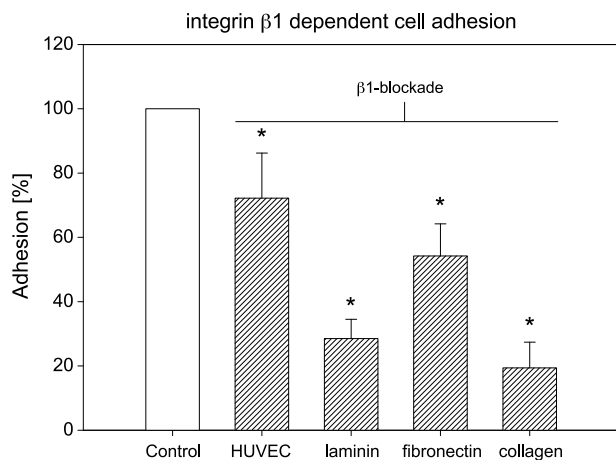
HCMV^{Hi} induced signalling
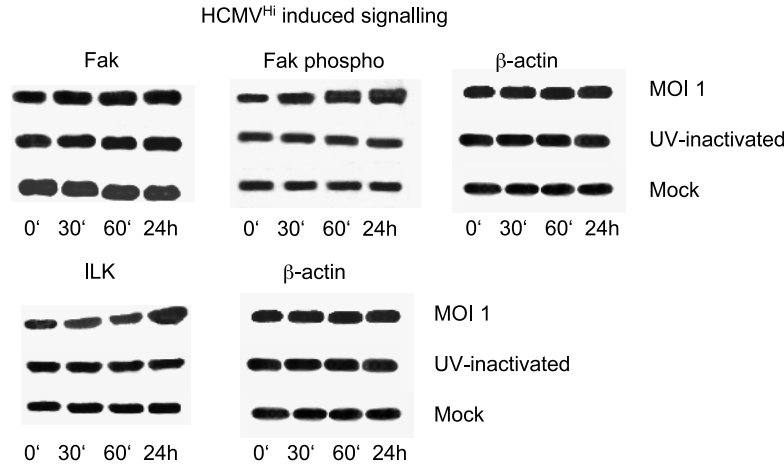
Figure 6. *HCMV^{Hi} modulates ILK, FAK, and FAKphospho. Mock-infected controls, HCMV^{Hi}-infected cells (MOI = 1), and UV-inactivated PC3 were examined by appropriate monoclonal antibodies, as indicated in the Materials and Methods section. β-Actin served as internal control. One of three representative experiments is shown. The x-axis indicates the time after HCMV^{Hi} infection.*

istics when compared to controls. In line with this observation, the expression of integrin β_1 subtypes did not change in transfected *versus* nontransfected PC3 cells (data not shown). However, an examination of oncogenic proteins revealed distinct differences in c-myc content among PC3 variants (Figure 9). Fluorometry in double-stained PC3^{pHM127} cells presented evidence of elevated c-myc levels, compared to nontransfected PC3 levels. Western blot assays demonstrate an enhanced c-myc content in all transfectants. The changes were most prominent in PC3^{pHM127} and PC3^{pHM135}, indicating that c-myc was mainly upregulated by IEA1.
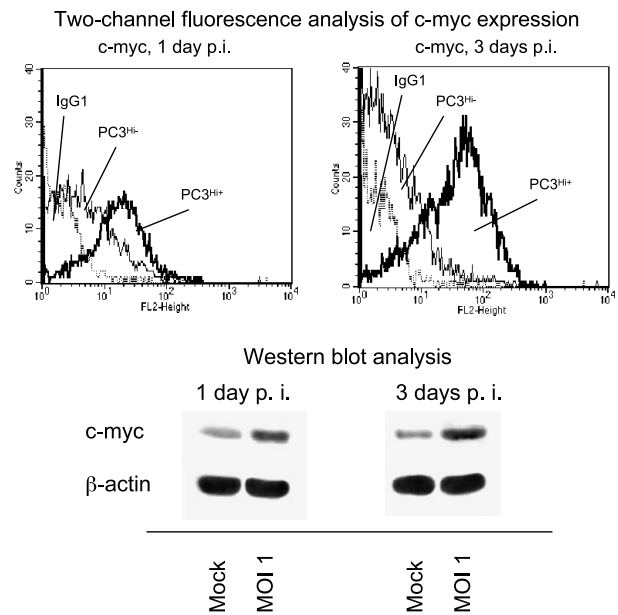
Discussion

Based on a cell culture model, we demonstrate for the first time that infection of PC3 cells with HCMV significantly alters

their invasive properties, as evidenced by enhanced tumor cell adhesion to the endothelium and extracellular matrix proteins. This discovery is fundamental to understanding HCMV's role in prostate cancer progression and supports the concept of cytomegalovirus-mediated oncomodulation [12]. Previously, we reported an enhanced invasiveness of
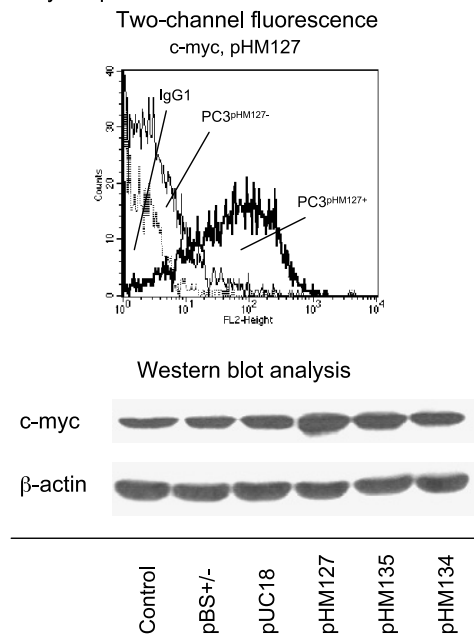
Two-channel fluorescence analysis of c-myc expression

Western blot analysis

Figure 8. *Two-channel fluorescence and Western blot analysis of c-myc. Top: HCMV^{Hi}-infected PC3 cell cultures were double-stained using, on the first step, a monoclonal antibody directed against the HCMV-specific 72-kDa IEA, labeled with FITC. On the second step, PC3 cells were marked with the PE-conjugated anti–c-myc monoclonal antibody. IEA$^-$ (PC3^{Hi-}) and IEA$^+$ cells (PC3^{Hi+}) were gated to obtain two distinct cell populations: population I (IEA$^+$) as HCMV-infected cells, and population II (IEA$^-$) as noninfected tumor cells. The c-myc expression of both PC3 subtypes was then detected by FACScan analysis [FL-2H(log) channel histogram analysis; 1 × 10^4 cells/scan]. To evaluate background staining, mouse IgG1–PE was used as isotype control. Bottom: Western blot analysis of c-myc from the proteins of HCMV^{Hi} (MOI = 1) versus mock-infected PC3 cells. Cell lysates were subjected to SDS-PAGE and blotted on the membrane incubated with c-myc monoclonal antibody. β-Actin served as internal control. The figure shows one of three representative experiments.*

Figure 7. *Adhesion of PC3 to HUVEC or extracellular matrix proteins is integrin-dependent. PC3 cells were preincubated with β_1 function–blocking antibodies (clone 6S6) and then added to HUVEC monolayers or immobilized collagen, laminin, or fibronectin. Adherent cells were counted after 60 minutes. The adhesion of cells not treated with monoclonal antibodies was set at 100%. Adhesion blockade diminished the adhesion to HUVEC and extracellular matrix proteins. One of three representative experiments is shown.*

Figure 9. *c-myc expression depends on the HCMV-specific immediate early protein IEA1. cDNA encoding HCMV IEA1 (UL123) was cloned into the $pBS^{+/-}$ vector and inserted in the expression vector pHM135. cDNA encoding HCMV IEA2 (UL122) was cloned into the $pBS^{+/-}$ vector and inserted in the expression vector pHM134. cDNA encoding HCMV IEA1 and IEA2 was cloned into the pUC18 vector and inserted in the expression vector pHM127. Control cells were transfected with vectors alone. The upper diagram presents a two-channel analysis of c-myc expression of pHM^+ versus pHM^- PC3 cell populations. Detailed information about cell staining is given in the Materials and Methods section. The lower diagram presents Western blot analysis of c-myc protein expression level. β-Actin served as internal control. The figure shows one of three representative experiments.*

HCMV-infected neuroblastoma cell lines when compared to the invasive capacity of noninfected variants [8]. Harkins et al. [13] described the specific localization of HCMV nucleic acids and proteins to neoplastic cells in human colorectal polyps and adenocarcinomas. However, reinvestigation of a larger tumor collective did not confirm the association between carcinogenesis and the progression of colorectal cancer and HCMV infection [14]. It is therefore difficult to assess whether our observation represents a unique phenomenon or if tumor-promoting properties of HCMV are a general feature that might occur in most, if not all, tumor types.

HCMV infection led to a significant upregulation of β_1-integrin receptors on PC3 cells in our experiments. Furthermore, β_1-blocking antibodies inhibited tumor cell adhesion to the endothelium or matrix proteins. We therefore conclude that (1) integrins of the β_1 family coordinate the interaction between prostate tumor cells and the endothelium/extracellular matrix, and (2) HCMV's effects on PC3 cell adhesion are caused by the elevation of integrin β_1 receptors.

Several reports corroborate the role of integrin proteins as key mediators of adhesion, migration, and invasion. Scott et al. [15] showed that blocking antibodies to the β_1-integrin subunit inhibited the adhesion of PC3 cells to bone marrow endothelial cells by 64%. Notably, osteoblast-mediated PC3 tumor cell motility and invasiveness were accompanied by increased $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrin expressions [16]. These

observations are in line with our postulation of HCMV-triggered integrin enhancement. Nevertheless, we should be aware that changes in β_1-integrin surface localization and activity status might also determine the metastatic behavior of prostate tumor cells. Hypothetically, HCMV could also act on the affinity of integrin receptors.

Indeed, elevated levels of ILK, as well as the promotion of FAK phosphorylation, were also observed in our cell culture model. Both proteins serve as important downstream components of integrin-mediated signalling and are involved in prostate cancer progression and invasiveness [17,18].

In this context, ectopic expression of active ILK in mammary epithelial cells induced a dramatic reorganization of the actin cytoskeleton and promoted rapid cell spreading [19]. Cherubini et al. [20] demonstrated that tyrosine phosphorylation of FAK may represent the necessary step to switch on the motility and invasiveness program in β_1-integrin–expressing tumor cells. It therefore seems likely that integrin overexpression, combined with the modulation of integrin-dependent signalling, is responsible for the acquisition of a more invasive—and thus more malignant—phenotype of $PC3^{Hi}$ tumor cells.

Novel data also support a critical role for $\alpha_2\beta_1$ and $\alpha_6\beta_1$ integrins as HCMV entry receptors [21]. Monoclonal antibodies to integrin subunits blocked both direct virus entry and HCMV gene expression. Cells lacking β_1 integrins were deficient in both entry and cell–cell spread of the virus, and the restoration of β_1-integrin expression in the same cell line restored both phenotypes. It is assumed that integrins serve as HCMV coreceptors that interact with the epidermal growth factor receptor (EGFR) to induce coordinated signalling. The coordination between integrins and EGFR seems to be crucial for successful viral infection [22]. Along with the role of integrins as adhesion regulators, HCMV probably establishes a symbiotic relationship between viruses and tumor cells. A high integrin expression level guarantees a high infection rate and simultaneously allows more tumor cells to become invasive (which may also serve as a mechanism of survival and escape from the host immune system). From a clinical viewpoint, the positive feedback mechanism between HCMV infection and integrin upregulation might dramatically accelerate prostate cancer dissemination and progression.

Nevertheless, we did not evaluate further adhesion receptors in our study. Therefore, receptors different from β_1 integrins may also be altered during HCMV infection and may be involved in virus-induced tumor cell adhesion events. This includes cadherins, selectins, CD44 receptors, or receptors of the CAM family.

The HCMV-induced modulation of neuroblastoma cell invasion was accompanied by distinct alterations of c-myc protein expression [8]. We consequently investigated whether this mechanism might also apply to the pathogenesis of prostate cancer. Indeed, the level of c-myc protein, an important oncogene in prostate cancer, was significantly increased in $PC3^{Hi}$ cells, compared to that in controls. A series of investigators has reported that c-myc amplification is present in up to 50% of high-grade prostatic intraepithelial neoplasia and 70% of primary prostate cancer, and that c-myc

amplification, with increasing Gleason score, increases with transition from prostatic intraepithelial neoplasia to localized prostate cancer to metastases [23].

Nevertheless, c-myc data are difficult to interpret, as the role of this protein in human cancer is complex and as no clear conclusion can be drawn about its definitive function in regulating cell adhesion. Our observation that UV-inactivated viruses did not lead to any changes in c-myc, although cell adhesion was enhanced significantly, does exclude a direct link between c-myc expression and tumor invasion. Several reports point to c-myc as a stimulator of cell cycle. Transgenic mouse models overexpressing c-myc in the prostate exhibited a dose-related progression toward malignancy. The resulting tumors resembled a poorly differentiated advanced carcinoma with accelerated growth rate, evidenced by $Ki67^+$ cells [24]. It is speculated that amplification of c-myc may have two explanations. At early stages, c-myc can confer a proliferative advantage by immortalizing prostate cells and by allowing them to grow under limited growth factor conditions. At later stages, c-myc may contribute to androgen-independent growth of prostate cancers. Based on this, HCMV-induced c-myc elevation might be sufficient to modulate carcinogenic events per se, but might not be directly coupled to the enhancement of adhesion processes.

According to the observation of Wang et al. [22], the attachment of UV-irradiated HCMV particles to PC3 cells evoked a distinct response, characterized by enhanced tumor cell binding to HUVEC and extracellular matrix proteins. This phenomenon clearly indicates that a physical association between the virus and host molecules (without virion delivery) has been established, reflecting an important event during HCMV-evoked oncomodulation and tumor dissemination. Even inactivated HCMV isolates might be sufficient to induce enhanced tumor cell invasiveness. Consequently, a therapeutic strategy that prevents both HCMV replication and docking at the tumor cell membrane is required. The underlying mode of action is not clear. Irradiated virus particles did not influence integrin expression level or ILK/FAK activity. Smith et al. [25] reported that a primary HCMV infection of human peripheral blood monocytes promoted transendothelial migration, increased cell motility, and upregulated adhesion molecule expression. UV-inactivated HCMV also evoked enhanced transendothelial migration, but not enhanced integrin expression, in this experiment. We therefore speculate that HCMV promotes tumor cell transmigration independently of viral gene expression.

In line with this speculation, transfection of PC3 cells with IEA1 or IEA2 cDNA did not induce any alterations of adhesion behavior and integrin expression, although c-myc was increased in these cells compared to controls. Shen et al. [26] showed recently that IEA1 and IEA2 evoke mutations in the *p53* gene in rat kidney cells. Furthermore, c-myc and *p53* were significantly elevated in endothelial cells or fibroblasts transfected with IEA1 and IEA2 plasmids [27,28], suggesting the oncogenic potential of IE proteins. Given the oncogenic activity of IEA1 and IEA2, it is not clear why they did not act on the tumor cell adhesion process in our culture system. One plausible explanation for this observation is that IEA1

and IEA2 only partially contribute to the invasive phenotype. Rather, a full infection scenario must proceed to alter the malignant properties of prostate cancer cells. Notably, HCMV attachment and infection at entry seem to play a crucial role in switching tumor cells from a low adhesive state to a high adhesive state. Wang et al. [22] pointed out that a cross-talk between EGFR and integrin receptors is necessary to allow successful HCMV infection and adequate downstream signalling. Indeed, blockade of integrin receptors inhibited HCMV virion content delivery and infectivity, while not inhibiting cell binding [22]. HCMV might, therefore, engage multiple receptors to form a multicomponent receptor complex and a functional signalling platform.

A second explanation is that IEA1 and IEA2 may promote malignant transformation by dysregulating various normal cellular physiological processes that specifically control cell cycle. IE proteins can interact with key regulatory proteins in the cell (e.g., members of the retinoblastoma family of proteins), resulting in the induction of DNA synthesis. The IE86 protein has also been shown to interact with the tumor-suppressor protein p53 (although we are aware that PC3 cells represent $p53^{-/-}$ mutants). In addition, the levels of the oncogenes *c-myc*, *c-fos*, and *c-jun*, as well as of cyclin E and cyclin-dependent kinases, are also rapidly upregulated following HCMV infection [29].

The elevated levels of c-myc found in IEA1-transfected or IEA2-transfected PC3 cell populations suggest that HCMV proteins might activate promoters involved in the regulation of cell proliferation, but might not directly modulate integrin-dependent tumor cell migration.

Based on our *in vitro* model, we postulate a direct association between HCMV infection and prostate tumor adhesion characteristics. HCMV shares the capacity of upregulating intracellular c-myc protein content in PC3 cells and evokes an elevated surface expression of β_1-integrin adhesion receptors, along with activation of downstream signalling. The latter effects are attributed, at least in part, to an enhanced invasive capacity of tumor cells. Further studies should explore the effects of antiviral therapy on prostate tumor growth and dissemination *in vivo*.

Acknowledgement

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