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The enhanced host-cell permissiveness of human cytomegalovirus is mediated by the Ras signaling pathway

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ABSTRACT

Human cytomegalovirus utilizes cellular signal transduction pathways to activate viral or cellular transcription factors involved in the control of viral gene expression and DNA replication. In the present study, we demonstrate that Harvey-*ras*-transformed cells show increased permissiveness to human cytomegalovirus when compared to their parental non-transformed cells. Both the progeny viral yield and the protein levels were elevated in the human cytomegalovirus-infected Harvey-*ras*-transformed cells requiring active viral gene replication, as shown by the infection with UV-inactivated human cytomegalovirus. Inhibition of Ras or of key molecules of the Ras pathway, effectively suppressed viral infection in the Harvey-*ras*-transformed cells. On a cellular level, the human cytomegalovirus-infected Harvey-*ras*-transformed cells formed larger cellular foci, which were significantly higher in number, compared to the uninfected cells and preferentially recruited human cytomegalovirus virions, thereby incriminating human cytomegalovirus infection for the increased transformation of these cells. Furthermore, proliferation assays revealed a higher rate for the human cytomegalovirus-infected Harvey-*ras*-transformed cells compared to mock-infected cells, whereas human cytomegalovirus infection had no considerable effect on the proliferation of the non-transformed cells. Higher susceptibility to apoptosis was also detected in the human cytomegalovirus-infected *ras*-transformed cells, which in combination with the higher progeny virus reveals a mode by which human cytomegalovirus achieves efficient spread of infection in the cells expressing the oncogenic Harvey-*ras* (12 V) gene. Collectively, our data suggest that human cytomegalovirus employs the host-cell Ras signaling pathway to ensue viral expression and ultimately successful propagation. Transformed cells with an activated Ras signaling pathway are therefore particularly susceptible to human cytomegalovirus infection.

1. Introduction

Human cytomegalovirus (HCMV, HHV-5) is a member of the β-herpesvidae family of viruses which can cause life-long persistent infections in the host [1,2]. The HCMV prevalence in the human population ranges between 50% and 85% and can be explained by its ability to remain latent for extended periods of time. Reactivation of the virus can be initiated by inflammatory or stress-related signals in the host organism, resulting in severe and occasionally fatal disease in immunocompromised individuals. Organ transplant recipients, pregnant women and AIDS patients are among the high-risk group due to their weakened immune system [3,4]. The reactivation of HCMV in healthy carriers is generally asymptomatic and without any consequences to the host, since these events are well controlled by the host cell-mediated immunity [5]. Human cytomegalovirus genomes are transcribed in a temporally defined fashion, and viral genes are classified with respect to their temporal expression, into Immediate Early, Early and Late.

The possible implications of HCMV infection to the host-cell oncogenic process have been investigated thoroughly, focusing on the presence of the virus in malignant tissues [6–8]. There are increasing data that suggest the presence of HCMV in several malignant tumors such as colon cancer [9], EBV-negative Hodgkin's lymphoma [10], prostatic carcinoma [11] and malignant glioblastoma [12,13]. In addition, HCMV is able to transform

Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; E, Early; EBV, Epstein-Barr virus; EGF, Epidermal Growth Factor; EGFR, Epidermal Growth Factor Receptor; ERK, Extracellular Regulated Kinase; FBS, Fetal Bovine Serum; FTI, Farnesyl Transferase Inhibitor; HFF, Human Foreskin Fibroblasts; HCMV, Human Cytomegalovirus; HPI, Hours Post-Infection; HHV, Human Herpes Virus; HSV, Herpes Simplex Virus; IE, Immediate Early; IPTG, isopropyl-1thio-β-D galactoside; L, Late; MAPK, Mitogen Activated Protein Kinases; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase; MOI, Multiplicity of Infection; NF-κB, Nuclear Factor kappa light chain enhancer of activated B cells; PAA, Phosphono acetic Acid; PFU, Plaque-Forming Units; PI3-K, Phosphatidylinositol 3-Kinase; PKR, RNA-activated protein kinase; RALGDS, Ral Guanine Nucleotide Dissociation Stimulators; SDS, Sodium-Dodecyl Sulphate; TNF α, Tumor Necrosis Factor; VZV, Varicella Zoster Virus

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human embryonic cells *in vitro,* showing enhanced tumorigenicity in these cells [14,15]. However, the expression of HCMV antigens decreased with increasing passage of the transformed cell lines, suggesting that the transformations occur in a "one-step" process and cease to affect the host cell therein[16,17]. It was then proposed that HCMV regulates the oncogenic process by indirect transformation events, termed as "onco-modulatory events", and does not affect the tumor cell directly like the Gamma-herpesviruses Epstein–Barr and Kaposi Sarcoma-associated herpesvirus [18].

In normal human fibroblasts, HCMV infection activates cellular proto-oncogenes and kinases involved in cell division and cell survival pathways, including c-myc, c-fos, c-jun, MAPK, ERK1/2 and PI3-kinase [19–23]. As regards the MAP kinase pathway, ERK kinases are activated by HCMV infection through the activation of MAP kinase kinases and inhibition of phosphatases [24]. A consequence of the MAP kinase activation by HCMV is the activation of transcription of viral genes, increasing the expression of viral gene products [25]. In particular, ERK kinases contribute to the activation of viral genes by cellular transcription factors, acting through the viral UL4 promoter at upstream and basal transcription elements. Although the implication of the downstream of Ras effector molecules, MEK and ERK, is well documented, the role of the *ras* gene, as the first step of the signaling cascade, has not been explored. The expression of oncogenic *ras* in human teratocarcinoma cells which induces differentiation, results in the conversion of the cells from a non-permissive to a permissive state for HCMV infection [26]. In addition, three different cell lines transformed by HCMV have been shown to harbor an activating mutation in both alleles in H-*ras*[27]. However, in both cases, it is unclear whether the mutations in H-*ras* were a direct result of the mutagenic activity of HCMV gene products. It is widely known that mutations in the *ras* family genes are found in approximately 30% of all human tumors, placing these genes in the forefront of cancer research [28–30]. Considering the capability of HCMV to activate members of the MAPK kinase pathway in combination with the increasing evidence for the implication of the virus in several oncogenic procedures, we sought to investigate the role of the oncogenic H-*ras* (12 V) in the context of HCMV infection. We demonstrate increased permissiveness and viral gene expression in constitutively activated H-*ras* cells compared to their parental non-transformed cells while the inhibition of Ras alone or key molecules of the Ras pathway, effectively suppressed viral infection. HCMV infection induces a higher proliferation rate of the H-*ras*-transformed cells and the formation of cellular foci as well as a preferential localization of the virions onto them. Taken together, these data suggest that HCMV exploits the Ras signaling pathway in a way that alters physiological cell proliferation and differentiation.

2. Materials and methods

2.1. Cells

The preneoplastic 208F Fischer rat fibroblasts [31], a morphologically flat subclone of NIH3T3 Swiss Embryo fibroblasts and FE-8, which is a tumorigenic cell clone derived from the 208F cells by co-transfection of the human EJ-RAS gene and the pSV2neo selection marker [32] were used in this study. FE-8 cells were cultured in the presence of G418 (400 μg/ml) as a selection marker) (Geneticin; GIBCO). Inducible *Ras* cells (IR-4) containing the inducible H-*ras* (12 V) were generated by stable transfection of 208F cells with the plasmids pSVlacOras and pH_lacINLSneo [33]. For the H-*ras* expression, 20 mM isopropyl-1thio-β-D galactoside (IPTG) was added to the medium and the IR-4 cells were incubated for 72 h. Human glioblastoma–astrocytoma epithelial-like U373MG cells were also used in this study to test our hypothesis in a human cell line [34–36].

The 208F, FE-8 and IR-4 cell lines (kindly provided by R. Schäfer, Charite, Universitaetsmedizin, Berlin) as well as U373MG cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Biosera) supplemented with 10% Fetal Bovine Serum (FBS; Biosera), Penicillin (100 units/ml and Streptomycin (100 μg/ml) unless otherwise stated. Human Foreskin Fibroblasts (HFF) were used for the propagation and titration of HCMV stocks. Cells were incubated at a 37 °C and a 5% CO_2 humidified atmosphere.

2.2. Virus

The AD169 strain of HCMV was used in this study. HCMV stocks were centrifuged, ultrafiltrated and titrated as previously described [37]. Infections of 208F and FE-8 cells were performed at multiplicity of infection (MOI) of 1 plaque-forming unit (pfu) per cell, i.e. MOI = 1 or MOI = 0.1 while U373MG cells were infected with HCMV at MOI = 3. The UV-inactivated virus was produced after UV irradiation (254-nm wavelength) of the active virus stock for 30 min at a distance of 20 cm [38]. Viral stocks were grown in Human Foreskin Fibroblasts (HFF) cells and titrated by plaque assays as previously described [39].

Infections in the absence of serum were carried out as previously described [23]. Briefly, the virus was first pelleted at 13,000 rpm for 2 h at 4 °C. The pellet was then gently washed and resuspended in serum-free medium and pelleted as described above for 1 h. Finally, the virus pellet was resuspended in serum-free medium, dimethyl sulfoxide (DMSO) was added to 1%, and the virus was stored at −80 °C. For infections in the presence of the farnesyl transferase inhibitor (Zarnestra), MEK inhibitor (UO126, Millipore; Bilerica, USA) and PI3K inhibitor (LY294002, Invitrogen; Camarillo, USA), cells were serum-starved for 24 h and pre-treated with drugs for 1 h prior to infection. Cells were then stimulated by dropwise addition of FBS and infected by dropwise addition of the virus. All drugs were solubilised in DMSO and all dilutions were made such that each dish of cells received an equal volume of DMSO. Cells were maintained in each drug for the duration of the experiment.

2.3. Release of infectious virions

The shedding of HCMV infectious virions from drug-treated or untreated 208 F, FE-8 and IR4 cells was investigated by a standard plaque-forming assay. Supernatant samples were collected from cell cultures and stored at −80 °C. Subsequently, an end-point dilution method was employed as follows: logarithmic dilutions were used to inoculate monolayers of HFF in three parallel series. After absorption for 2 h, the inoculum was replaced with normal culture medium containing anti-HCMV serum. Infections were allowed to progress until viral plaques were observed. The results from supernatant samples were expressed as PFU per milliliter. The assays were performed three times and the averages of the results ± one standard deviation are presented.

2.4. Viral nucleic acid isolation and analysis

For real-time PCR, DNA was extracted from virus-infected cells at 8, 24, 96 and 144 hours post-infection (h.p.i.), using the DNeasy tissue kit (QIAGEN, Hilden, Germany). Quantification of viral input DNA by real-time PCR was performed using the Q-CMV Real Time Complete Kit (Nanogen Advanced Diagnostics, Italy). For each sample, DNA extracts were analyzed in triplicate.

2.5. Retroviral infection

2×10^6 HEK293T cells were transfected using TurboFect (Fermentas, USA) with 8 μg of retroviral vector pBabe-H-Ras^{V12} (courtesy of Stephane Ansieau, Lyon, France) [46]. pBabe-puro was used as a control retroviral vector. Forty-eight hours after the transfection, the supernatant was harvested, filtered and aliquots were stored at −80 °C. The supernatant was applied to 10^6 U373MG human glioblastoma–

astrocytoma epithelial-like cells overnight in the presence of 8 μg/ml Polybrene (Sigma-Aldrich, USA). Infected cells were selected after 48 h using Puromycin (0.5 μg/ml) (Sigma-Aldrich, USA).

2.6. Plasmids and transfection assays

The pEGFP-C2 plasmid was purchased from Clontech Laboratories Inc., USA. The plasmid pEGFP-IE1 was generated after fusion of the *ie1* gene derived from the pGEX-3X-IE1 [40] to the Clontech vector pEGFP-C2. The expression vector pEGFP-IE2 [41] has been described previously. For transfection experiments, 208F or FE-8 cells were seeded either on glass coverslips or into four-well, chambered coverglass units with coverslip quality glass bottoms (Lab-Tek; Nunc). For transient expression assays, DNA (1 μg/well) was introduced in subconfluent cells using the TransPEI transfection reagent (Eurogentec, Belgium) according to the manufacturer's instructions.

2.7. Proliferation assay

To measure the effects on cell proliferation over time due to HCMV infection, total cell count assays were performed [42]. Briefly, 2×10^6 208F and FE-8 cells were plated in 6-well plates in the absence of FBS and were subsequently infected with wt-HCMV, UV-irradiated HCMV or mock infected at MO1 = 1. The cells from duplicate plates were trypsinized 3 days post-infection, resuspended in DMEM and counted using a hemacytometer. Cells were stained using Trypan Blue (Sigma-Aldrich, USA) dye at 0.8 mM, which was mixed at equal (1:1) parts with suspended cells. All proliferation experiments were performed at least three times. Additionally, 2×10^6 U373MG cells were plated in 6-well plates and subsequently infected with wild-type HCMV, UV-irradiated HCMV or mock infected. The cells from duplicate plates were trypsinized 3 days post-infection, resuspended in DMEM and counted using a hemacytometer. Furthermore, U373MG cells transduced with either the pBabe or pBabe-H-Ras^{V12} retroviruses were plated in 6-well plates and infected with HCMV (MOI = 3) to quantify their proliferation at 1, 3 and 5 days after the infection. Cells were resuspended in DMEM and counted using a hemacytometer. Cell counting was carried out by Trypan Blue (Sigma-Aldrich, USA) dye staining as described above.

2.8. Focus formation transformation assays

To determine whether HCMV could modulate the focus forming activity of the activated H-*ras* (12 V), FE-8 cells were infected with HCMV at MOI = 1 in 6-well plates. The cells were then maintained in DMEM supplemented with 1% calf serum. The appearance of transformed foci of cells was quantitated 3–5 days post-infection [43]. Similarly, the human U373MG cells were transduced with retroviruses expressing either the pBabe-H-Ras^{V12} or the empty pBabe vector and subsequently were infected with HCMV at MOI = 3. Images were taken 3 weeks after the infection to visualize foci formation. In some experiments, the Ras inhibitor (FTI R115777), the MEK1/2 inhibitor (PD098059), the PI3K inhibitor (LY294002) and the HCMV inhibitor (PAA) were added to the medium at a concentration of 200nM, 50 μM, 200 μg/ml and 1 μM, respectively. All assays were performed in triplicate.

2.9. In vitro wound healing assay

FE-8 cells were seeded in 24-well culture plates at a concentration of 80,000 cells per well. The cells were cultured in an incubator at 37 °C and 5% CO_2 and 24 h after plating, they were either infected with HCMV (M.O.I. = 1) or mock infected. The cell layer was wounded by scratching with a sterile 10 μL pipette tip after 48 h of infection. Detached cells were removed by washing two times with serum-free medium. The wound closure was monitored at 0, 3, 6 and 9 h.p.i., using a digital image processor connected to an inverted microscope. Cell motility was quantified by image analysis (ImageJ 1.4.3.67 Launcher Symmetry Software). Each experiment was carried out in triplicates and the error bars represent the mean standard error.

2.10. Indirect immunofluorescence analysis

For indirect immunofluorescence analysis, 2×10^5 208F and FE-8 cells were plated on glass coverslips placed in 24-well plates. The medium was aspirated, the cells were washed with phosphate-buffered saline (PBS) and HCMV was added at MOI = 1 and the mixture was incubated at 37 °C for 2 h. The inoculum was subsequently removed, fresh medium was added and the cells were incubated for 72 h. The cells were then fixed, permeabilized and indirectly stained for the viral pp65 protein, using the CMV pp65 Antigenemia Antibody kit by Light Diagnostics (Temecula, CA).

2.11. Apoptosis assays

Apoptosis was evaluated by applying both caspase-3 staining and a DNA fragmentation assay. 2×10^5 FE-8 cells were plated on glass coverslips placed on 24-well plates, infected at MOI = 1 with HCMV and then incubated for 2 and 3 days post-infection. At the above time points the cells were fixed and stained with a caspase-3 rabbit polyclonal antibody (Santa-Cruz Biotechnology Inc., sc-7148) at a dilution of 1:200, as well as with an IE1 mouse monoclonal antibody (BS500) at a dilution of 1:50. After primary incubation, cells underwent secondary staining for 1 h with secondary conjugated antibodies, rabbit Cy3 (Invitrogen) and the mouse Alexa-488 (Invitrogen) at a dilution of 1:500, respectively. The coverslips were then inspected under an epifluorescence microscope.

For DNA fragmentation analysis, mock and HCMV-infected FE-8 cells were harvested 1, 2 and 3 days post infect and resuspended in a solution containing 10 mM Tris, pH 8, 10 mM EDTA, and 150 mM NaCl. Cells were then incubated with 0.1 mg/ml proteinase K and 1% SDS at 50 °C overnight. The samples were extracted in phenol-chloroformisoamyl alcohol and ethanol precipitated, and the pellets were washed with 70% ethanol and dried. The pellets were resuspended in Tris–EDTA (TE), pH 7.5. The DNA was electrophoresed in a 3% agarose gel and ethidium bromide stained [39].

2.12. Western blotting

For Western blotting, cell lysates from HCMV-infected and mock-infected cells were harvested using the M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA), enriched with the Halt-Protease Inhibitor Cocktail, EDTA-free (PIERCE, Rockford, IL, USA) according to the manufacturer's instructions. Proteins were separated on 12% polyacrylamide gels containing Sodium-Dodecyl Sulphate, and then transferred to nitrocellulose membranes. The Western blotting and chemiluminescence detection were performed as previously described [44]. The HCMV IE1 and UL44 gene products were detected using mouse monoclonal antibodies against IE1-72KDa (BS500) and ppUL44 (BS510) as previously described [45]. Mouse monoclonal anti-actin antibody was used at a dilution of 1:4000 (Pierce, Rockford, IL, USA); while secondary anti-rabbit (AP132P) and mouse (AP124P) conjugated Horseradish Peroxidase antibodies were purchased from CHEMICON (Temecula, CA, USA) and used at a dilution of 1:5000.

3. Results

3.1. Increased viral expression and progeny virus in H-ras-transformed cells

In view of the significance of several oncogenes in the outcome of herpesvirus infection, we first investigated the role of the oncogenic

H-*ras* in the permissiveness of HCMV. Parental 208 F cells and FE-8 cells transformed with H-*ras* (12 V) as well as IR-4 cells which inducibly express activated H-*ras* were infected with HCMV, at MOI = 0.1 and MOI = 1. More specifically, the growth properties of progeny virus were assessed by single-step growth curve analyses by collecting supernatants from infected 208 F and FE-8 cells at time points corresponding to 1, 3, 5 and 7 days post-infection. The viral yield from the abovementioned time points was significantly increased in the FE-8 cells when compared to the parental 208F cells, at both MOI = 0.1 and MOI = 1, peaking at seven days post-infection (Fig. 1A, a and b). Consistently, supernatants collected from infected IR-4 cells, produced an increased viral yield when the cells were treated with IPTG, which induces H-*ras* (12 V) expression, as opposed to when they were not treated with IPTG. We observed that the viral yield in the IPTG-induced IR-4 cells was increased both at low and high MOI, showing a peak at seven days post-infection, similar to the results we observed in the 208F and FE-8 infected cells (Fig. 1A, c and d).

Additionally, we sought to determine the viral particle-to-PFU ratios between 208 F and FE-8 at different time points of the infection. For this purpose, DNA was extracted from 208F and FE-8 cells at 8, 24, 96 and 144 hours post-infection and quantified by real-time PCR (Fig. 1B). The viral particle uptake was equivalent between the 208F and FE-8 cells, as indicated by the amount of viral DNA detected 8 h.p.i. Interestingly, the number of viral DNA copies significantly increased during the course of the infection in the H-*ras*-transformed cells when compared to the 208F cells in a manner similar to the produced progeny virus we observed earlier. These data suggest that an activated Ras signaling pathway confers a functional advantage for the DNA replication of HCMV.

The permissiveness to HCMV was also assessed at the level of viral protein synthesis. The expression levels of indicative HCMV proteins

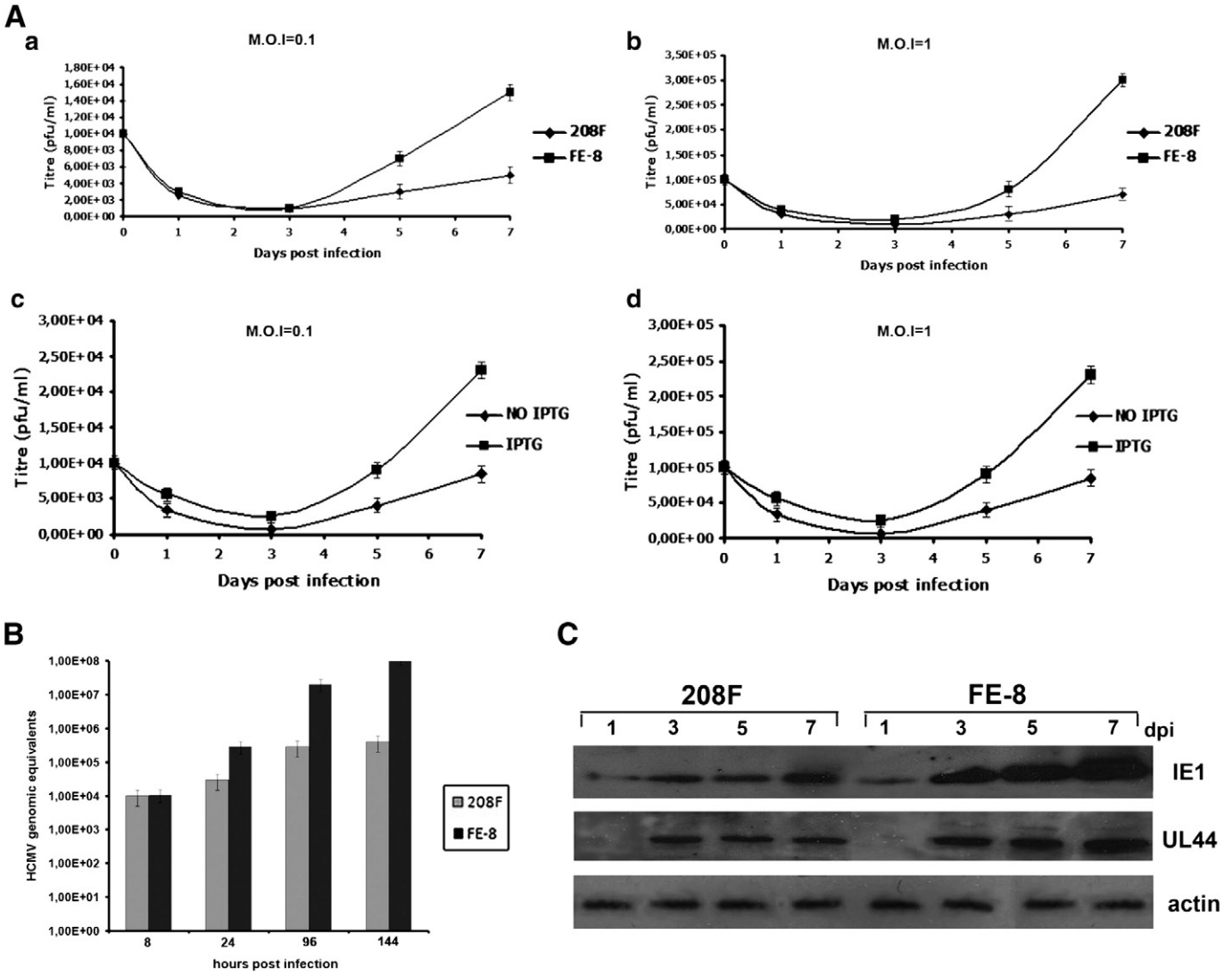

Fig. 1. Increased HCMV expression and progeny virus in H-*ras*-transformed cells. A: Single-step growth analysis of infected 208 F and FE-8 cells. a, b, 208 F and FE-8 cells were infected with HCMV AD169 at MOI = 0.1 and MOI = 1. Supernatants were collected at 1, 3, 5 and 7 days following infection and the viral titers were evaluated and represented as plaque-forming units per ml of virus (pfu/ml). Error bars represent the standard deviation derived from three independent experiments. c and d, Inducible Ras (IR-4) cells were infected with wild-type HCMV strain AD169 at MOI = 1 and MOI = 0.1. IR-4 cells were either stimulated with IPTG to induce H-*ras* (V12) expression, or not treated with IPTG. Supernatants were harvested at different time points post-infection as indicated, followed by quantification of the observed viral plaques. B: 208F and FE-8 cells were infected with HCMV AD169 at MOI = 1 and DNA was extracted at 8, 24, 96 and 144 h following infection. Quantitative real-time PCR results were used to evaluate the input of HCMV genome copies. C: Comparison of HCMV protein expression between infected 208 F and FE-8 cells. Cells were harvested at the indicated time points after infection and the proteins separated by 12% SDS-PAGE gel electrophoresis. Immediate Early HCMV protein IE1 and the Early UL44 protein were analyzed by Western blotting. Cellular actin levels served as a control for equal protein loading.

such as the immediate early protein IE1 as well as the late protein UL44 were examined by Western blot analysis (Fig. 1C). The infection of FE-8 cells by HCMV resulted in an increasing expression pattern for both viral proteins peaking on the seventh day post-infection. On the contrary, the viral protein expression in the infected parental 208F cells, for both IE1 and UL44, remained at basal levels throughout the time course of the infection, raising more questions on the role of HCMV infection in the H-*ras*-transformed cells. Additional experiments using HCMV-permissive [36] human glioma cells U373MG indicated that HCMV infection is enhanced in cells that express the mutated form of Ras. The expression levels of IE1 and UL44 were observed by Western blot of U373MG cell lysates at 1, 3 and 5 days post-infection. Cells retrovirally expressing the mutated H-Ras^{V12} showed an increased expression of viral proteins at 3 and 5 d.p.i. when compared to cells that did not express the mutant Ras protein (Supplementary Fig. S1).

Taken together, these results demonstrate that the Ras signaling pathway plays an important role in HCMV infection.

3.2. Inhibition of HCMV infection by Ras inhibitors

The role of *ras* genes during normal cellular function is dictated by the post-translational modifications to which the proteins are subjected. Farnesylation, the main Ras modification, precedes the plasma membrane anchorage of Ras proteins which in turn determines their status, active or inactive [47]. If Ras, is in fact involved in HCMV infection, then inhibition of the farnesylation should block HCMV replication in the transformed cells. Treatment of HCMV-infected *ras*-transformed cells with the farnesyl transferase inhibitor FTI, resulted in a markedly reduced progeny virus compared to the non-treated cells (Fig. 2A). The low viral yield of the FTI-treated cells was comparable to the blockage caused by the known HCMV inhibitor phosphonoacetic acid (PAA). These results are consistent with the idea that HCMV infection requires an activated Ras signaling pathway.

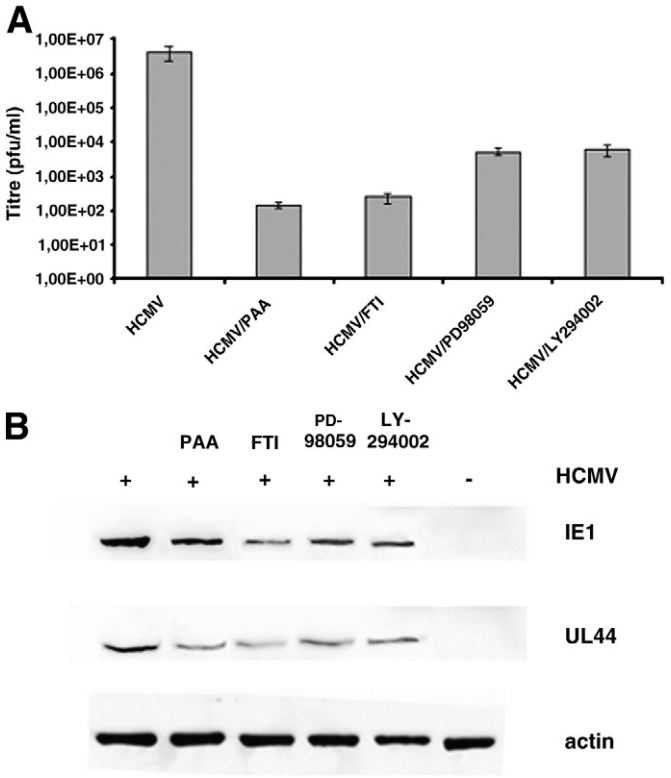

Fig. 2. Inhibition of HCMV infection by Ras inhibitors. A: Titration of HCMV virion shedding by plaque assay quantification. FE-8 cells were infected with HCMV (MOI = 1) or treated with PAA, FTI, MEK inhibitor and PI3K inhibitor prior to infection. Supernatants from drug-treated FE-8 cells were subjected to plaque-forming assay as described in the Materials and methods section. Viral plaque formation was assessed and expressed as pfu/ml of virus stock. Infection and plaque assay experiments were performed in triplicate. B: Western blotting analysis using antibodies against viral IE1 and UL44 proteins. FE-8 cells were pre-treated with four inhibitors (PAA, FTI, MEK, PI3K) and subsequently were infected with HCMV (MOI = 1) and harvested at 3 days post-infection. Actin served as a control for equal protein loading.

GTP-bound RAS is able to bind and activate effector enzymes of important pathways downstream of Ras and among others, the Ras-Raf-MEK-ERK-MAP kinase cascade and the PI3K pathway [48]. To determine whether MEK1/2 or PI3K activities are required for HCMV infection, we studied the effect of the PD098059 and LY294002 inhibitors, respectively, on the infected *ras*-transformed cells. HCMV titration assays showed that both inhibitors also blocked viral replication in the *ras*-transformed cells. However, the effect was not that striking compared to FTI. The effects of the above inhibitors on the progeny virus were also mirrored by their effects on the viral protein synthesis as compared to the non-treated *ras*-transformed cells (Fig. 2B). The concentrations of all drugs used did not inhibit the overall protein synthesis (data not shown). Collectively, our results strongly suggest the implication of an active Ras signaling pathway for a productive HCMV infection, while the downstream ERK and PI3K pathways are partially involved in HCMV infection.

3.3. Effect of HCMV infection in H-ras-transformed and non-transformed cells

HCMV modulates important properties of the host, such as cell cycle regulation or apoptosis [7]. Considering the above regulatory effects of HCMV on the host, we sought to investigate potential changes of the proliferation rate and motility properties of *ras*-transformed and non-transformed infected cells. For this purpose, 208F and FE-8 cells were infected with HCMV at MOI = 1, mock infected, or infected with UV-irradiated HCMV. The proliferation characteristics of the infected cells were significantly different, depending on the *ras* status. The HCMV-infected parental 208F cells exhibited only a slight increase in the number of cells compared to the mock-infected cells (Fig. 3A). Strikingly, the HCMV-infected FE-8 cells exhibited a significant increase in the proliferation rate 3 days post-infection, in comparison to the non-infected cells ($p<0.05$). Infection of both *ras*-transformed and non-transformed cells with the UV-inactivated HCMV had no major effect on the growth properties of the cells, providing evidence that the adhesion of the virus alone is not sufficient, but active viral gene expression is required for the increased proliferation of the H-*ras* transformed cells. Similar experiments in U373MG cells that were either transduced with pBabe-H-Ras^{V12} or with the empty pBabe vector, were carried out by infecting them with HCMV, UV-inactivated HCMV or mock infected. Interestingly, the proliferation of the pBabe-H-Ras^{V12} transduced U373MG cells that were infected by HCMV was increased significantly at 3 d.p.i when compared to the empty vector-transduced cells or the UV-HCMV-infected cells (Supplementary Fig. S2A). Furthermore, the retroviral expression of H-Ras^{V12} combined with HCMV infection conferred to the cells a proliferative advantage over time (Supplementary Fig. S2B).

In addition, an *in vitro* "wound healing" assay demonstrated lower migration efficiency of the HCMV-infected compared to the mock-infected *ras*-transformed cells. Our results showed that HCMV-infected FE-8 cells closed 32.2% ± 1.9% of the inflicted wound area 9 h post-infection, as compared to the 16.4% ± 1%closure of the mock-infected cells at the same time point (Fig. 3B and C). These data clearly shows that HCMV enhances the migration capacity of the *ras*-transformed cells, augmenting their oncogenic potential.

3.4. Formation of cellular foci upon infection of H-ras-transformed cells

Oncogenic forms of RAS are locked in their active state and transduce signals essential for several oncogenenic processes including cellular

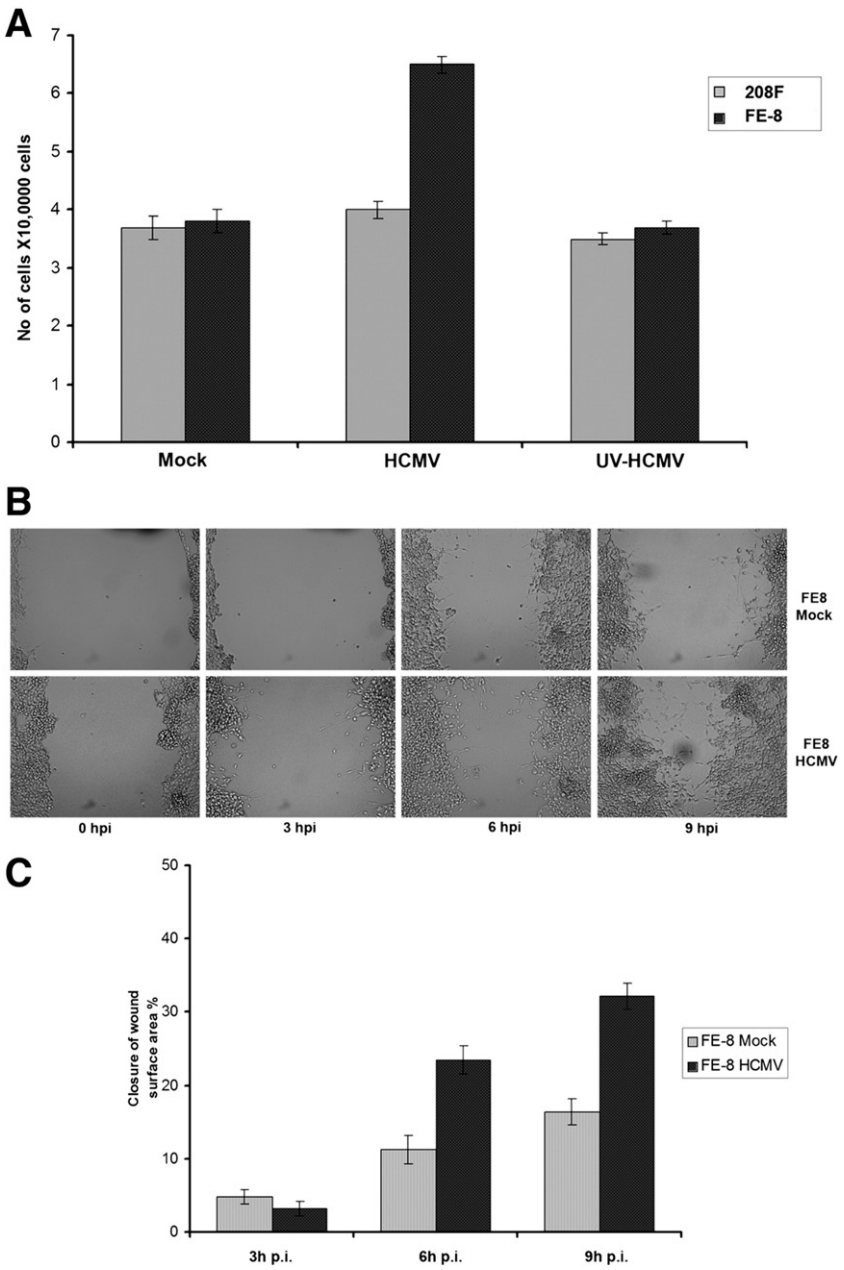

Fig. 3. Effect of HCMV infection in H-*ras*-transformed and non-transformed cells. A: Comparison of proliferation rate between HCMV-infected 208F and FE-8 cells. The cells were initially serum-starved and subsequently were either mock-infected, infected with wild-type HCMV or UV-irradiated HCMV at MO1 = 1. Cell counting was performed on viable cells, stained with Trypan Blue, using a hemacytometer. B: FE-8 cells were plated in 6-well plates and infected with HCMV or mock infected. The monolayer was "wounded" 48 hpost-infection with a 10 μl sterile pipette tip and the detached cells were removed. Images were taken at 0, 3, 6 and 9 h after the scratch to monitor cell motility. C: Quantification of wound healing was performed using the ImageJ image analysis software. The results were expressed as % of closure of wound surface area at different time points (at time point 0 h the wound surface area was the 100%).

proliferation and transformation activating downstream pathways involving the RAF/MEK/ERK cascade of cytoplasmic kinases, the phosphatidylinositol 3-kinase and others [49,50]. In the 208F/FE-8 system, the preneoplastic 208F cells do not transform spontaneously, whereas the malignant FE-8 cells are anchorage-independent [51]. To further investigate the impact of HCMV on the transformation properties of V12 H-*ras*-activated cells, 208F and FE-8 cells were either infected by HCMV or mock infected and the cells were observed at 5 days post-infection. In our experiments, inefficient foci formation was observed in both HCMV and mock-infected parental 208 F cells (Fig. 4A, a and b). On the contrary, HCMV-infected FE-8 cells exhibited a remarkable proliferation potential, forming cellular foci that were significantly larger in size (over 200 μm) and number (160 ± 6 foci) compared to the mock-infected cells (45 ± 3 foci) ($p < 0.05$) (Fig. 4A, c and d). Moreover, human glioma cells U373MG were transduced with either the pBabe-H-Ras^{V12} retroviral vector or the empty vector, followed by HCMV infection and allowed to incubate for several weeks. The cells were regularly monitored for foci formation and to observe differences between the retroviral H-Ras^{V12} expressing cells as well as the HCMV-infected and mock-infected cells (Supplementary Fig. S3). The U373MG cells, in the absence of retroviral H-Ras^{V12} expression did not exhibit any cellular focus after 3 weeks of incubation, regardless the HCMV infection (Supplementary Fig. S3 a and b). Consistently with the aforementioned experiment in FE-8 cells, HCMV infection resulted in the development of higher number of cellular foci in

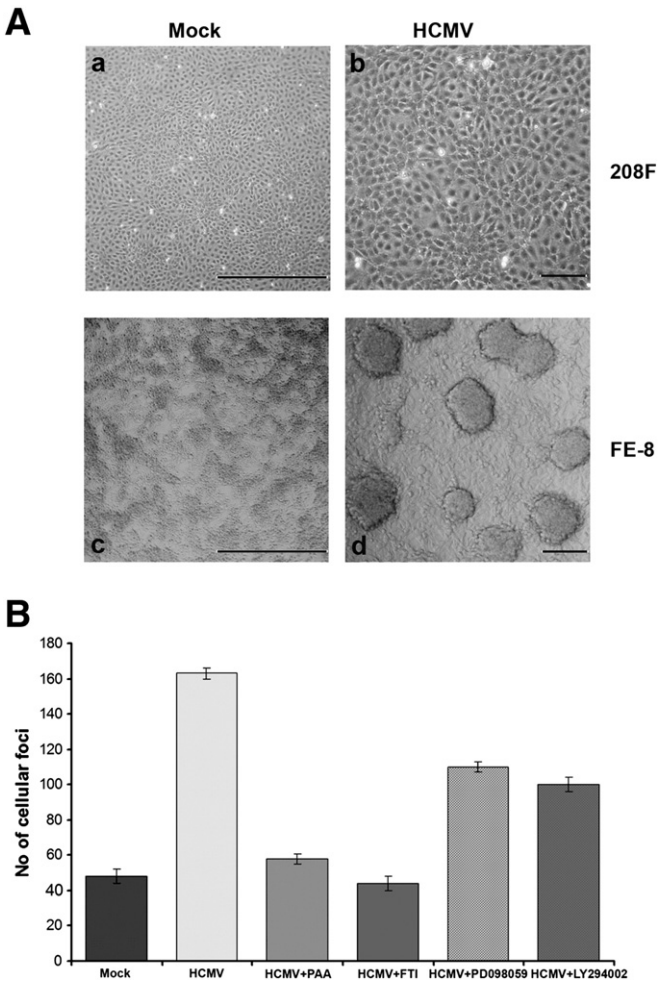

Fig. 4. Formation of cellular foci upon infection of H-*ras*-transformed cells. A: 208 F (a, b) and FE-8 (c, d) cells were plated in 6-well plates and either mock infected or infected with HCMV at MOI = 1. Cellular foci were observed at 3–5 days following infection (bars, 10 μm). B: FE-8 cells were treated with PAA, FTI, MEK inhibitor or PI3K inhibitor, 1 h prior to infection with HCMV. The cellular foci of FE-8 cells were quantitated at 5 days after infection. Foci formation assays were performed in triplicate. Error bars indicate deviations between experiments.

the cells overexpressing the mutated form of H-Ras (Supplementary Fig. S3 c and d). These results support the previously reported oncomodulatory role of HCMV which is incapable of transforming cells on its own. However, it significantly augments the transformation efficiency of constitutively activated H-Ras (12 V) cells.

To further explore the role of Ras/MEK/ERK and Ras/PI3K/Akt pathways in the formation of the above cellular foci, we performed inhibitor assays on HCMV-infected FE-8 cells and evaluated the number of cellular foci forming at 5 days post-infection (Fig. 4B). Inhibition of the viral infection by the HCMV replication inhibitor phosphonoacetic acid (PAA) reversed the cellular foci formation bringing it closer to the mock-infected numbers. The involvement of Ras in the cellular foci formation upon HCMV infection was investigated by the addition of the farnesyl transferase inhibitor (FTI), which not only reversed the foci formation but accomplished a slight reduction in foci numbers compared to the mock-infected cells. Finally, the addition of MEK (PD098059) and PI3K (LY294002) inhibitors resulted in a partial reversion of the cellular foci formation, indicating the probable involvement of these Ras effector molecules in the transformation properties of FE-8. Similar results were obtained when the above experiment was repeated in the human glioblastoma U373MG cell line after retroviral transduction (data not shown).

3.5. Preferential localization of HCMV virions onto ras-transformed cellular foci is not due to IE1 and IE2

In order to further verify the additive effect of HCMV on the focus formation properties of the H-*ras*-transformed cells, the localization of the newly synthesized viral particles was investigated in both the 208F and FE-8 cells. The viral structural protein, pp65, was used as a marker for the detection of the progeny virions. Immunofluorescence staining for the aforementioned viral protein demonstrated that pp65 was relatively evenly distributed among the infected 208F cells, indicating an overall infection of these cells (Fig. 5A, b). Interestingly, the viral protein was found to be almost exclusively localized on the cellular foci (Fig. 5A, d). Although the H-*ras* transformed cells did not form a typical flat monolayer in the cell culture however, analysis of higher magnification images confirmed the preferential localization of pp65 onto the cellular foci (Fig. 5A, f). The striking association of the viral protein onto the foci of FE-8 cells further supports our hypothesis that HCMV infection induces the formation of the cellular foci and enhances the transformation capabilities of the H-*ras*-transformed cells.

In efforts to delineate a potential function of HCMV in the neoplastic process of H-*ras*-transformed cells, we investigated biological properties emanating from its immediate early gene products IE1 and IE1, which are key regulators of virus replication and survival [52,53]. For this purpose, FE-8 cells were transiently transfected with plasmids expressing either the IE1 protein (pEGFP-IE1) or the IE2 protein (pEGFP-IE2). The empty vector plasmid pEGFP-C1 was used as the control, to verify possible foci formation. The overexpression of either IE1 or IE2 gene products did not result in any distinct morphological alteration in the FE-8 cells (Fig. 5B). Neither IE1 nor IE2 induced focus formation within 5 days post transfection, indicating the absence of primary transforming activity. Moreover, selected mass populations did not exhibit colony formation in semi-solid medium, showing lack of anchorage-independent growth. Given the high sensitivity of FE-8 cells in detecting transforming properties, the above findings directing oncogenic activity were not associated with either IE1 or IE2 proteins.

3.6. Enhanced apoptosis in HCMV-infected H-ras-transformed cells

HCMV infection is known to block the apoptotic process of the host-cell, by affecting death receptor signaling pathways, such as the TNF-mediated death receptor signaling pathway [54,55]. Therefore, we investigated the effects of HCMV infection on the H-*ras*-transformed cells and whether the *ras* oncogene plays a role in the process of apoptosis. Caspases are a protein family of proteases that play a crucial role in the programmed cell death [56,57]. Caspase-3 in particular is a good indicator for investigating cellular apoptosis. Consequently, FE-8 cells were either mock infected or infected with HCMV and fluorescently labeled for the viral protein IE1 and caspase-3. The mock-infected FE-8 cells did not exhibit any caspase-3 expression at 2 days, whereas there was minimal caspase-3 activation at 3 days of culture. Interestingly, the HCMV-infected FE-8 cells showed significant caspase activation even at 2 days post-infection, a phenomenon that peaked at 3 days post-infection (Fig. 6A).

Moreover, whole cell extracts of HCMV and mock-infected FE-8 cells were harvested at 1, 2 and 3 days, followed by DNA extraction. The DNA extracts were then loaded on an agarose gel and visualized by ethidium bromide staining, for DNA fragmentation patterns. DNA fragmentation is one of the final steps of apoptotic cell death, where DNA cleavage produces DNA fragments of various sizes. In our experiment the mock-infected FE-8 cells exhibited basal levels of DNA laddering, whereas the effect was more distinct on the HCMV-infected cells, indicating an increase in DNA degradation at 2 and even more at 3 days post-infection (Fig. 6 B).

4. Discussion

Human cytomegalovirus initiates infection and intracellular signaling by binding to its cellular cognate receptors and by activating several signaling pathways, including those mediated by PI3K [21], mitogen-activated protein kinases [20,22,58] and G proteins [59]. In the present study, we examined the involvement of host-cell Ras signaling pathway in human cytomegalovirus infection. Our experiments indicated a direct implication of the Ras protein in the progress of HCMV infection. Both the progeny virus and viral expression levels were significantly enhanced in the H-*ras*-transformed cells, whereas non-transformed cells (208F) or cells with inactivated oncogenic Ras (IR4) restrained viral infection to basal levels. Inhibition of the Ras protein by the farnesylation inhibitor (FTI) resulted in decreased viral progeny verifying a functional link between HCMV infection and Ras protein expression. Molecules downstream of Ras, such as ERK or PI3K were also involved but in a lower extent suggesting that the activated Ras protein poses a major role for a productive HCMV infection. Herpes Simplex Virus type-1 (HSV-1) but also its mutant versions or the oncolytic herpes G207 have been shown to exploit the activated Ras signaling pathway to increase viral permissiveness by inhibiting the virus-induced activation of the stranded RNA-activated protein kinase (PKR) [60–62]. Additionally, reovirus also takes the advantage of the activated Ras signaling in the host cell, resulting in increased viral permissiveness by suppressing the IFN-β production through negative regulation of RIG-I signaling [63–65] while the RAS/Raf1/MEK/ERK signaling pathway also facilitates VSV-mediated oncolysis [66,67]. Apparently, HCMV has also evolved mechanisms to usurp an already activated signal transduction pathway, the Ras signaling pathway, rendering the cells more susceptible to viral infection.

Emerging evidence suggests that HCMV infection and expression may be specifically associated with human cancers by deregulating signaling pathways involved in the initiation and promotion of malignancy [8,68,69]. One significant finding of this study was the increased proliferation rate of the HCMV-infected H-*ras*-transformed fibroblasts, when compared to their parental non-transformed cells, which is justified by the activation of the Ras protein [70]. Strikingly, the proliferation rate of both cell lines was increased when they were infected by HCMV, while the transformed cells exhibited the highest levels of proliferation and cell motility. H-*ras* transformed fibroblasts seem to provide an ideal environment for HCMV replication, exploiting the host-cell Ras/Raf/MEK/ERK pathway to regulate and increase the proliferation rate of the infected cells. The Immediate Early protein IE1 plays a crucial role in viral DNA replication and acts as a precursor protein for the expression of subsequent viral proteins [71,72]. In addition, the Early UL44 protein is essential to HCMV DNA replication because it acts as an accessory protein to the viral DNA polymerase [73,74]. The expression levels of both proteins were notably elevated in the *ras*-transformed fibroblasts establishing a direct association between the host-cell Ras protein and HCMV. However, the function of these particular viral proteins does not appear to directly affect the proliferative status of neither the transformed nor the non-transformed cells.

H-*ras* (G12V) transformed cells (FE-8) tend to accumulate in clusters of cells, forming cellular foci [51,75]. This characteristic which is expected in transformed cells was profoundly enhanced by HCMV infection. Remarkably, HCMV proteins were almost exclusively localized in these cellular foci, increasing the transformation potential of FE-8 cells, whereas the presence of a HCMV inhibitor (PAA), or the MEK and PI3K inhibitors reversed the cellular foci formation close to that of uninfected cells. It is possible that HCMV infection provides selective pressure for tumor cell populations with increased

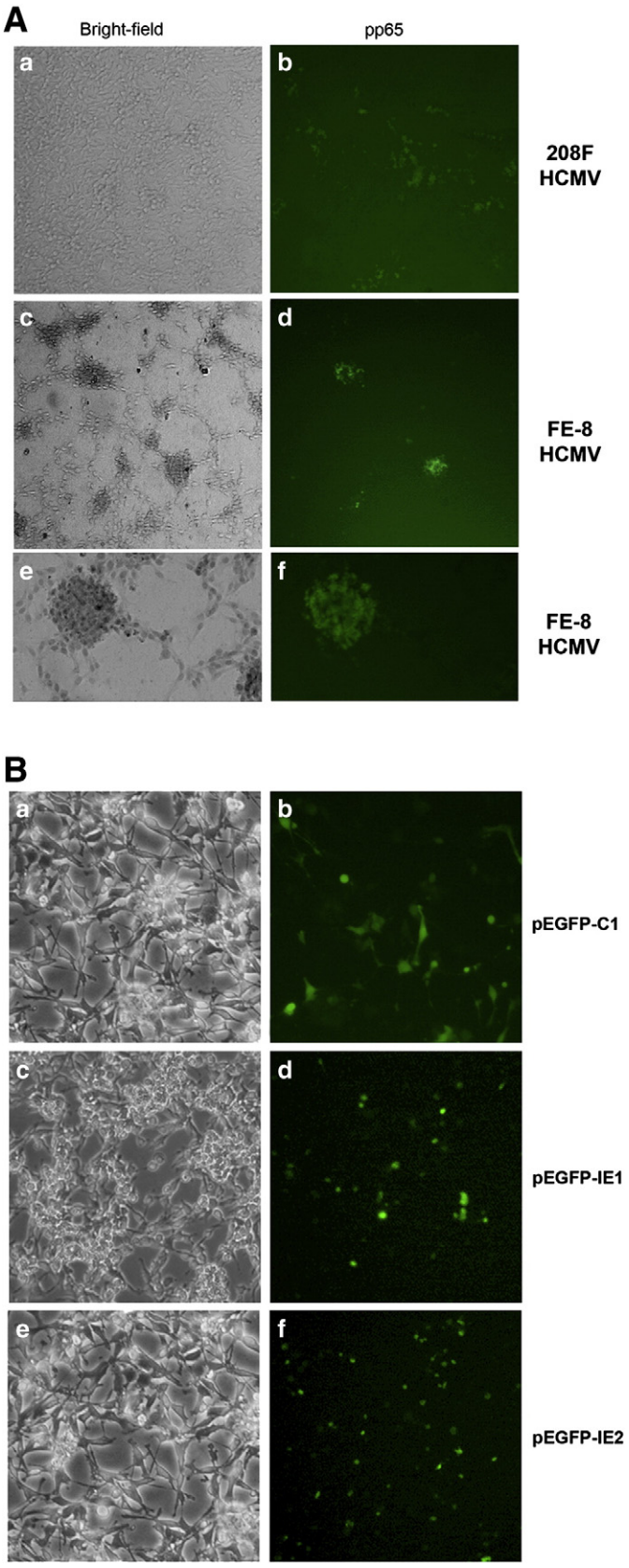

Fig. 5. Preferential localization of HCMV virions onto *ras*-transformed cellular foci but is due to IE1 and IE2. A: 208 F (a, b) and FE-8 (c–f) cells were plated on glass coverslips and were either mock infected or infected with HCMV at MOI = 1. The viral tegument protein pp65 was stained by indirect immunofluorescence as described in the Materials and methods section. B: FE-8 cells were seeded into four-well, chambered units with coverglass quality glass bottoms. 1 μg/ml plasmid DNA was transfected to the cells for transient expression of IE1 (c, d), IE2 (e, f) or pEGFP-C1 (a, b). The EGFP signal was observed on an epifluorescence microscope 72 h following transfection.

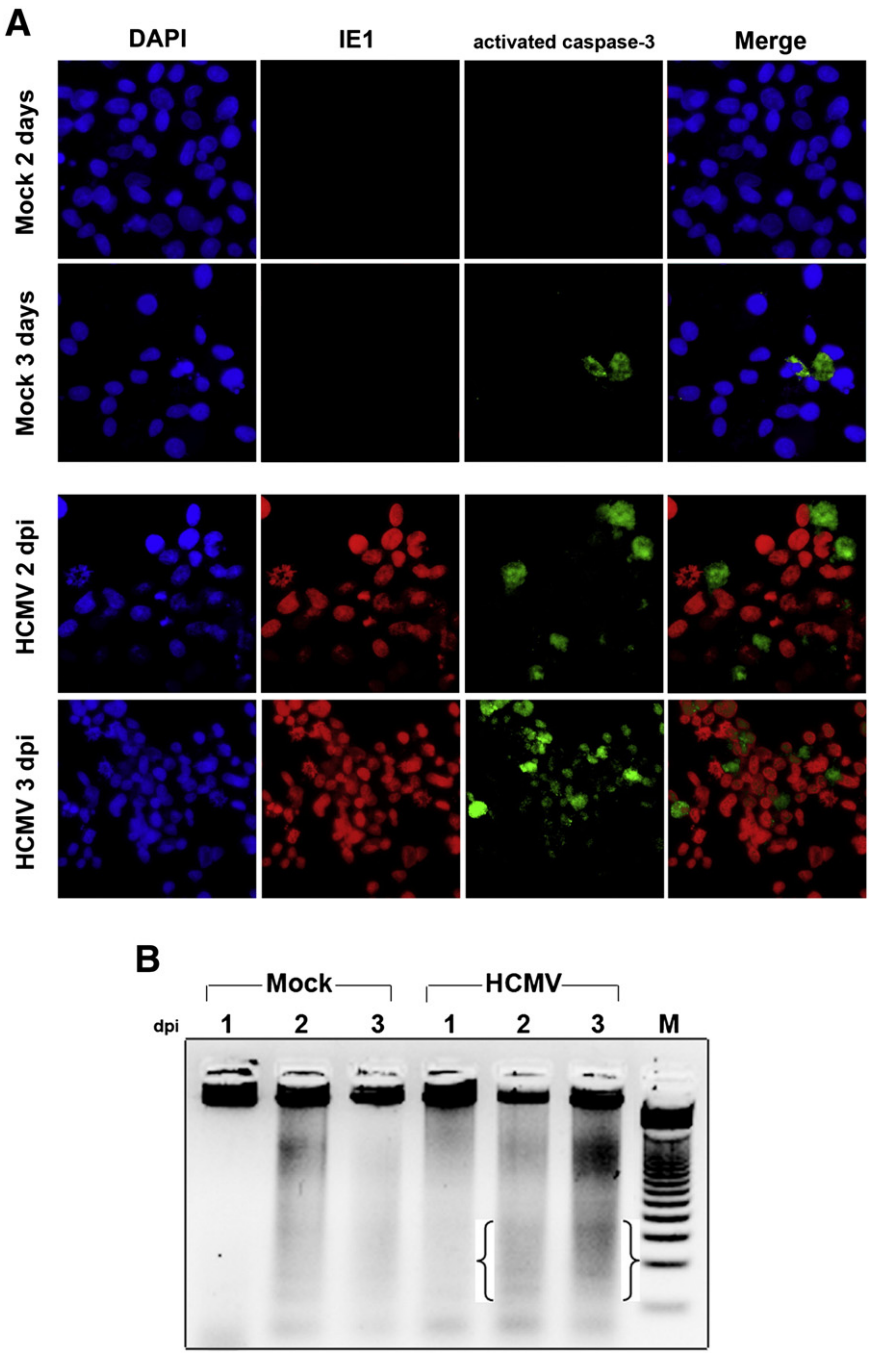

Fig. 6. Enhanced apoptosis in HCMV-infected H-*ras*-transformed cells. A: FE-8 cells were plated on glass coverslips, mock-infected or infected with HCMV at MOI = 1 and then incubated for 2 and 3 days post-infection. Cells were stained for IE1 (red color) and for activated caspase-3 (green color). B: Both infected and uninfected FE-8 cells were harvested at 1, 2 and 3 days post-infection. Genomic DNA was isolated and subsequently electrophoresed on a 3% agarose gel. Small DNA fragments mainly between 250 and 750 bp yielded a "ladder" pattern on the agarose gel (see brackets). M: 1Kb DNA ladder.

malignant potential. Infection of malignant cell lines by HCMV has been associated with increased transformation of these cells [16,27]. Although HCMV does not directly transform cells *in vitro*, data acquired from viral infection of transformed cells indicate that the overall genetic microenvironment of the malignant cell enables HCMV to further upset the balance [18]. Therefore, it could be suggested that the activated Ras signaling pathway facilitates the establishment of a favorable tumor microenvironment where HCMV is capable to modulate normal cellular functions such as cell proliferation or motility and finally promote a malignant phenotype.

A key property of nearly all tumor cells is the resistance to apoptosis and the attenuation of cell proliferation and differentiation. The HCMV Immediate Early protein IE2 is known to suppress apoptotic cell death in the host-cell, at least until successful viral replication takes place. During HCMV infection, the expression of IE2 is responsible for the inhibition of p53, TNF-α and Doxorubicin-induced apoptosis [76–78]. HCMV protects malignant cells from apoptosis, by activating the cellular proteins AKT, Bcl-2 and ΔNp53α [79]. As regards the Ras protein and apoptosis, the activation of Ras modulates the expression of several genes involved in apoptosis [80]. The oncogenic Ras can either promote or inhibit apoptosis, depending on the cell type and the nature of the apoptotic stimuli. It is known that the oncogenic *ras* (12 V), is able to induce apoptosis in colon cancer cells, by increasing the sensitivity of the cells to 5-FU-induced

apoptosis [81] or in response to sulindac [82] or other chemotherapeutic agents [83]. Additionally, there are several proapoptotic genes that are downregulated in human tumors and could explain the increased apoptosis in fibroblasts carrying the oncogenic *ras*[84]. Interestingly, the investigation of the effect of HCMV infection in apoptosis using this particular oncogenic Ras model, revealed an increased susceptibility to apoptosis of the H-*ras*-transformed cells compared to their parental cells. Apparently, the previously proapoptotic properties of the oncogenic Ras (12 V) dominate over the antiapoptotic action of HCMV, tilting the balance towards apoptotic cell death. Coupled with the higher viral titers detected in the H-*ras*-transformed cells, we make the key connection between apoptosis, which leads to enhanced virus release and efficient spread of infection in *ras*-transformed cells. Alternatively, the infected H-*ras* transformed cells could advance to apoptosis in order to inhibit the unusually high proliferation rate induced by HCMV.

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