

Original Paper

The Role of RhoA, RhoB and RhoC GTPases in Cell Morphology, Proliferation and Migration in Human Cytomegalovirus (HCMV) Infected Glioblastoma Cells

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Key Words

HCMV • RhoA • RhoB • RhoC • Glioblastoma cells • Cell migration • Cell proliferation • IE1

Abstract

Background/Aims: Rho GTPases are crucial regulators of the actin cytoskeleton, membrane trafficking and cell signaling and their importance in cell migration and invasion is well-established. The human cytomegalovirus (HCMV) is a widespread pathogen responsible for generally asymptomatic and persistent infections in healthy people. Recent evidence indicates that HCMV gene products are expressed in over 90% of malignant type glioblastomas (GBM). In addition, the HCMV Immediate Early-1 protein (IE1) is expressed in >90% of tumors analyzed.

Methods: RhoA, RhoB and RhoC were individually depleted in U373MG glioblastoma cells as well as U373MG cells stably expressing the HCMV IE1 protein (named U373MG-IE1 cells) shRNA lentivirus vectors. Cell proliferation assays, migration as well as wound-healing assays were performed in uninfected and HCMV-infected cells. **Results:** The depletion of RhoA, RhoB and RhoC protein resulted in significant alterations in the morphology of the uninfected cells, which were further enhanced by the cytopathic effect caused by HCMV. Furthermore, in the absence or presence of HCMV, the knockdown of RhoB and RhoC proteins decreased the proliferation rate of the parental and the IE1-expressing glioblastoma cells, whereas the knockdown of RhoA protein in the HCMV infected cell lines restored their proliferation rate. In addition, wound healing assays in U373MG cells revealed that depletion of RhoA, RhoB and RhoC differentially reduced their migration rate, even in the presence or the absence of HCMV. **Conclusion:** Collectively, these data show for the first time a differential implication of Rho GTPases in morphology, proliferation rate and motility of human glioblastoma cells during HCMV infection, further supporting an oncomodulatory role of HCMV depending on the Rho isoforms' state.

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Published by S. Karger AG, Basel

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Introduction

The Rho GTPase family, a relative of Ras proto-oncogenes, consists of more than 20 proteins in humans, divided into subfamilies including Rho, Rac, Cdc42, RhoH, RhoBTB, Rho, Rnd and Rif (Rif and RhoD) [1]. Rho GTPases have been implicated in a variety of cellular processes and most importantly of cytoskeleton organization and its impact on biological functions on cellular movement and division [2, 3]. Cdc42, Rac1 and RhoA are the far well-characterized members of the superfamily. In addition, recent studies have shown that Cdc42, Rac1 and RhoA have a modulatory role in cellular trafficking and tumorigenesis [4]. Like other GTPases, the Rho GTPases cycle switches between the active GTP-bound form and inactive GDP-bound form. The guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) are the key regulators of the Rho GTPase superfamily [5]. GEFs mediate the dissociation of GDP and the recruitment of GTP, activating GTPases. GAPs facilitate Rho GTPase activation by stimulating Rho GTPases intrinsic GTP hydrolyzing activity, resulting in a GDP-bound form. The third category of Rho GTPase family regulators, the Rho GDIs, retain the GTPases in an inactive form through their binding to C-terminal prenyl groups on Rho proteins [6]. Through the interconversion between the inactive GDP-bound and active GTP-bound conformational states, Rho proteins are important molecular regulators of cellular functions such as cell proliferation, shape, polarity, adhesion and migration, vesicle trafficking, differentiation and transformation [7-9].

To establish metastasis in distant tissues, tumor cells have to enter the vascular or lymphatic system, then exit it and proliferate in a new tissue. The ability of Rho GTPases to regulate cytoskeletal dynamics, cell adhesion and cell migration [10] marks a central role for these proteins in cancer cell invasion and metastasis. The highly conserved RhoA, RhoB and RhoC proteins are frequently aberrantly expressed in human tumors, with RhoA and RhoC being frequently overexpressed whereas RhoB is often downregulated [11]. RhoA has been implicated in all stages of cancer progression and has an important role during tumour cell proliferation, survival and progression, controlling the generation of epithelial polarity, junction assembly and disruption of epithelial cells [12]. Furthermore, RhoA is important for both amoeboid and mesenchymal migration through the activation of the RhoA-ROCK signaling pathway [13]. On the other hand, RhoC limits to metastasis in cancer progression [14]. Studies of RhoC deficient mice show that RhoC is required for metastasis but not for the tumour initiation [14]. Besides, the expression of RhoB has been associated with tumor aggressiveness while it is often downregulated in human tumors [15]. RhoB has been proposed to act as a tumour suppressor inhibiting tumour growth, cell migration and invasion [15]. RhoB deficient mice have a normal developmental profile but display oncogenic formation [16].

Human cytomegalovirus (HCMV) is a double-stranded DNA virus that belongs to the family of Human Herpesviruses. It is a beta herpes virus that establishes a lifelong latent infection after a primary infection [17]. HCMV has the largest genome among all herpes viruses, approximately 230 kbp that is divided into a unique large (UL) and unique short (US) region. HCMV encodes over 200 open reading frames, but the exact number is depending on the strain [18]. It is known for its opportunistic infection in immunocompromised individuals such as in AIDS patients, organ and stem cell transplant patients and it is the main etiological agent responsible for congenital diseases in newborn babies [19]. Replication of HCMV commences in the nucleus of host cells and is regulated by the expression of the immediate-early (IE) genes which are regulatory genes controlling early and late genes expression in infected cells [20]. HCMV IE genes 1 and 2 (IE1 and IE2) are the first set of viral genes that are activated in infected cells. IE1 and IE2 proteins regulate transcription of viral and cellular genes during HCMV infection and have been implied in the pathogenesis of many diseases [21].

HCMV proteins have been identified in several types of solid tumors, such as malignant gliomas, neuroblastomas, medulloblastomas, prostate, colon, breast and lung cancer [22-25]. Glioblastoma multiforme (GBM) is the most aggressive brain tumour in humans and is regulated by many different molecules [26, 27]. The microenvironment of glioblastomas seems to be restricted when the expression of KIF14 is inhibited [28] or due to low doses given of curcumin [29]. Furthermore, the tumour growth and progression of gliomas has been shown to be diminished in case of inhibition of autophagy in late stages [30]. In more than 50% of these tumours, HCMV proteins are highly expressed in tumor tissues. Due to this association between HCMV and cancer, an oncomodulatory role for HCMV has been proposed [31-33]. Stable expression of HCMV IE1 protein has been shown to differentially enhance or arrest glioblastoma cell growth and promote cell cycle entry and DNA synthesis, indicating that IE1 can modulate the oncogenic phenotype of human glioma cells and its expression in tumor cells activates signaling pathways associated with cancer. [34]. Furthermore, HCMV IE1 can promote glioblastoma stemness, cell-cycle progression and survival demonstrating a novel role of IE1 as potent driver for glioblastoma stem-like cells [35] and revealing that HCMV infection might promote pathogenesis in gliomas [36]. In addition, there are studies demonstrating that the IE1 expression increases the proliferation rate in primary glioblastoma cells via the suppression of p53 protein and the activation of Akt signaling [37-39] inducing the expression of a negative p53 protein regulator [40]. Beyond, the increase of telomerase activity is also correlated with IE1 gene expression in glioblastoma cell lines while there are reports displaying the co-localization of HCMV IE1 protein with hTERT proteins in gliomas [41]. Since it is well established that increased telomerase activity leads cells to be immortalized, this IE1 mediated enhancement of telomerase activity might modulate the microenvironment of tumour in glioblastoma cells [42].

The expression of HCMV glycoprotein G has also been detected in glioblastomas enhancing growth and invasiveness through the activation of PDGFR α [43]. Moreover, HCMV promotes neoplastic transformation [31] and induces mucoepidermoid proliferation by activating oncogenic signalling pathways [44]. HCMV has been also shown to inhibit apoptosis by regulating the activation of transcription factor 5 (ATF5) signaling pathway in human malignant glioma cells [45]. Persistence of HCMV in malignant glioma cells may result in a minimal cytopathic effect and therefore, HCMV may be reactivated in latently infected glioma cells when cells are exposed to inflammatory stimuli [46]. HCMV encodes for gene products that regulate cellular pathways involved in mutagenesis, apoptosis and host antitumour immune responses as their sustained expression edges to glioma constitution [47]. Throughout the years, human herpes viruses have employed genes encoding viral G protein-coupled receptors (vGPCRs), which are expressed in infected host cells. HCMV encodes four GPCRs [48] which have been modified to be used by the virus to take over the control of the host cell for its own benefit [49]. Rho GTPases which are activated through coupling of G proteins to GEFs in order to stimulate proliferation, differentiation, and inflammation in a variety of cell and types [50], are also used from HCMV for the interaction of viral components with cellular proteins. [51]. The engagement of viral proteins with the cell modulates the structure of the cytoskeleton actin and the function of actin effector molecules, such as Rho GTPases, to initiate infection and promote viral dissemination, which results in a high infection rate among the human population [52-55].

Given the growing interest in the role of CMV in cancer epidemiology, etiology, pathogenesis in combination with the active implication of Rho GTPases in human tumourigenesis, we aimed to investigate the role of Rho GTPases in HCMV permissive U373MG and their derivatives stably expressing IE1, U373MG-IE1 glioblastoma cells. Our results demonstrate an important role of RhoA, RhoB and RhoC in cell morphology both in the absence or presence of HCMV infection. Furthermore, the oncomodulatory role of HCMV was further revealed when the original inhibition in the proliferation rate of RhoA depleted cells was restored in the presence of viral infection.

Materials and Methods

Cells and virus

Primary Human Foreskin Fibroblasts (HFF), HEK-293T cells, the human glioblastoma cell line U373MG as well as its derivative U373MG-IE1 cell line stably expressing the HCMV IE1 protein were grown in DMEM (Gibco BRL) supplemented with 10% foetal bovine serum (Gibco BRL), 100 U/ml penicillin and 100 µg/ml streptomycin under 5% CO₂ in a humidified incubator at 37°C. HFF cells were used for the propagation and titration of HCMV virus. The stable expression of IE1 was maintained in U373MG-IE1 cells using G418 (1mg/ml) (Sigma-Aldrich, USA). Human embryonic kidney cell-line 293T (HEK-293T cells) was used only for the production of shRNA TRIPZ lentiviruses. The wild-type HCMV AD169 strain was used in this study. The virus stocks were propagated and titrated on HFF cells according to standard protocols [56]. For viral infections, the cells were infected with HCMV at the indicated MOI for 2 hours and then the inoculum was removed and replaced by fresh medium.

Lentiviruses Production

HEK-293T cells were transfected using 10 µl Fugene 6 (Promega) with 3 µg of either TRIPZshRhoA (RHS4852, Thermo Scientific), TRIPZshRhoB (V2THS_172671, Thermo Scientific) or TRIPZshRhoC (RHS4743, Thermo Scientific) vectors, 2 µg of pCMV-dR8.91 (Delta 8.9) plasmid and 1 µg of VSV-G, according to the manufacturer's protocol. The TRIPZ Inducible Lentiviral Empty Vector shRNA Control (RHS4750, Thermo Scientific) was used as a control lentiviral vector. The vectors above are engineered to be Tet-on and produce tightly regulated induction of shRNA expression in the presence of doxycycline (500 ng/ml). Additionally, in these vectors, turboRFP and shRNA are part of a single transcript, allowing the visual marking of shRNA-expressing cells. Forty-eight hours after the transfection, the supernatants were harvested, filtered and stored at -80°C. The supernatants were applied to U373MG and U373MG-IE1 cells in the presence of 8 µg/ml Polybrene (Sigma-Aldrich, USA). Transduced cells were selected after 48 hours using puromycin (1 µg/ml) (Sigma-Aldrich, USA) and maintained in the presence of this antibiotic.

Immunofluorescence Analysis

For immunofluorescence, 1 × 10⁵ U373MG cells, transduced with the empty vector (EV) lentivirus vector and induced with doxycycline, were plated on glass coverslips in 24-well plates. The cells were subsequently infected with the recombinant HCMV AD169/IE2-EGFP virus expressing IE2 fused to the enhanced green fluorescent protein [57] at MOI = 3 pfu/cell. The cells were fixed with formaldehyde (4% [vol/vol] in PBS containing 2% sucrose) and the nuclei were stained with DAPI. Fluorescent images were acquired with an epifluorescent Leica DMIRE2 microscope, equipped with a Leica DFC300FX digital camera.

MTT assay

MTT assay was performed to determine the cell proliferation rate of both the parental U373MG and the derivative U373MG-IE1 cells after the knockdown of RhoA, RhoB or RhoC proteins, either in the presence or absence of HCMV infection. Briefly, 2 × 10⁶ cells were transduced with either the shRNA Empty Vector or the TRIPZshRhoA or the TRIPZshRhoB or the TRIPZshRhoC lentiviruses, induced with doxycycline and subsequently were either infected with HCMV (MOI=3 pfu/cell) or mock infected to quantify their proliferation rate at 1 and 3 days after infection. In each cell type, the yellow tetrazolium MTT reagent (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (Sigma Alndrich, cat. no. R8755) was added and incubated for 4 hours at 37°C followed by the addition of 150 µl MTT solvent (dimethyl sulfoxide-DMSO) for 15 minutes and finally the measurement of the absorbance at 590 nm with a reference filter of 620 nm. All experiments were performed in triplicates.

In vitro wound healing assay

U373MG and U373MG-IE1 cells were transduced either with the shRNA Empty Vector or TRIPZshRhoA, TRIPZshRhoB or TRIPZshRhoC lentiviruses and were grown until high confluence in 6-well plates. Twenty-four hours after plating, the cells were either infected with HCMV (MOI=3 pfu/cell) or mock infected and the monolayer were wounded with a pipet tip after 48 h post infection. Cell debris was removed by washing two times with serum-free medium and monolayer maintained in medium for 72 hours with or without HCMV. The wound closure was monitored at 0, 3, 6, 9, 12, 24, 48 and 72 h.p.i, using an epifluorescent Leica

DMIRE2 microscope, equipped with a Leica DFC300FX digital camera. For each experimental point 8 fields photographed and the cells migrating inside 3 mm of wound were counted. Each experiment was carried out in triplicates and the cell motility was quantified by ImageJ 1.4.3.67 analysis (Launcher Symmetry Software).

Time Lapse Microscopy

In chambered coverglass units (Lab Tek, Thermo Scientific) 1×10^5 cells were seeded and infected at MOI 3 pfu/cell with HCMV AD169 virus. Forty-eight hours after infection, the cells were transferred in humidified chamber on the microscope stage with 5% CO₂ at 37°C. Still images from live cells were taken every 15 minutes for 5 hours using an epifluorescent Leica DMIRE2 microscope, equipped with a Leica DFC300FX digital camera. Images were exported as TIFF files and were processed by Photoshop.

Morphometry

For cell shape analysis cells were plated in two-well coverglass units chambers and bright field and fluorescent images of live cells were acquired with an epifluorescent Leica DMIRE2 microscope, equipped with a Leica DFC300FX digital camera. The total area was measured with Metamorph Cellprofiler Software (Genome Biology).

Statistical analysis

All data shown represent independent experiments carried out in triplicates. The measurements were compared by analysis of logged data (Graphpad Prism) and the significant differences were determined using a one-way ANOVA.

Results

Generation of glioblastoma cells with stable knockdown of RhoA, RhoB and RhoC

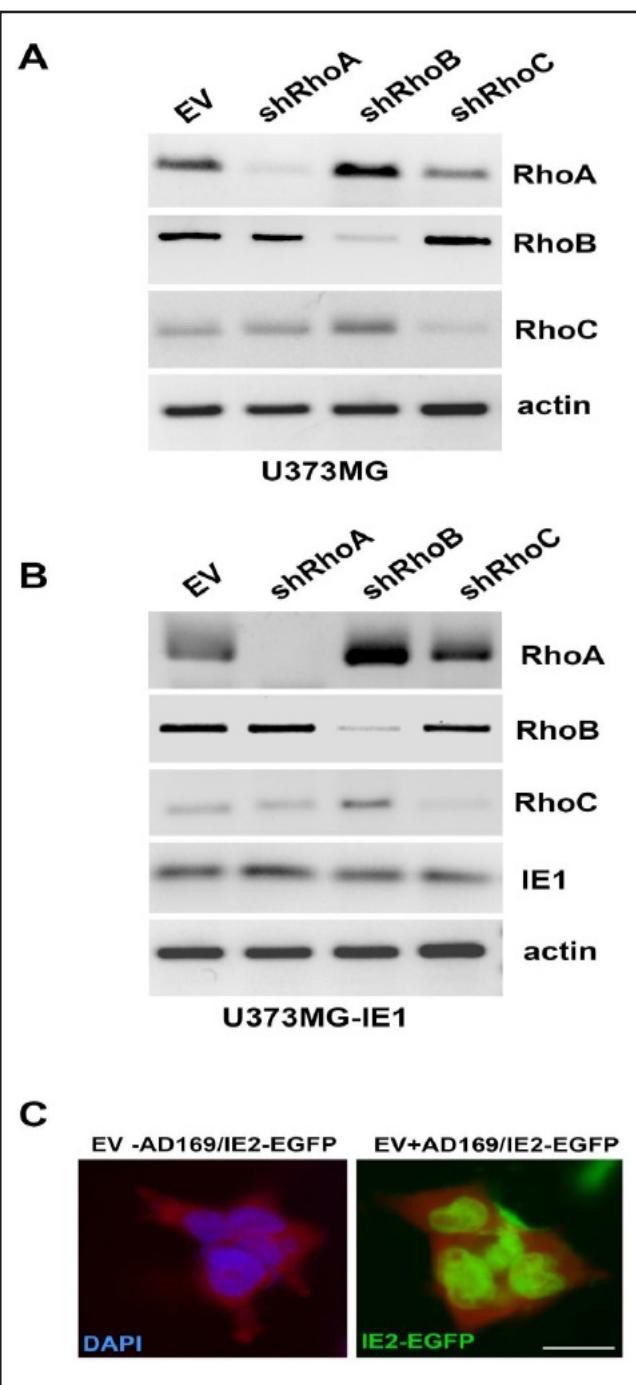
To investigate the role of RhoA, RhoB and RhoC in glioblastoma cells in the context of HCMV infection, we first established glioblastoma cells which were devoid of the above Rho GTPases. Parental U373MG cells and cells stably expressing the HCMV IE1 protein, named U373MG-IE1, were transduced with the doxycycline-inducible TRIPZ lentiviral vectors expressing shRNA specifically targeting RhoA, RhoB or RhoC. A doxycycline-inducible TRIPZshRNA lentiviral empty vector (EV) was also used, serving as a negative shRNA control vector. All lentivirus transduced cells were subsequently selected with puromycin. The efficiency of silencing for each Rho GTPase was determined by Western Blot in total cell extracts (Fig. 1). RhoA or RhoC depletion did not affect the expression of the other isoform however, RhoA and to a lesser extent RhoC silencing induced RhoB overexpression, which is in agreement with previous studies [58, 59]. On the contrary, RhoB knockdown did not affect RhoA or RhoC expression levels.

Cell morphology of RhoA, RhoB and RhoC knockdown glioblastoma cells in uninfected and HCMV infected cells

We next examined the morphological changes caused by the knockdown of RhoA, RhoB and RhoC in glioblastoma cells, in the absence or presence of HCMV. In non-infected U373MG cells, depletion of RhoA resulted in an elongated cell shape compared to the control cells (Fig. 2A). An analogous phenotype with long and thin protrusions was observed in RhoA-depleted U373MG-IE1 cells (Fig. 2B). Similarly, RhoC-knockdown cells led to elongated, mesenchymal-like cells (Fig. 2A and 2B), an observation which is consistent with the depletion of RhoC in other cell lines [60, 61]. Increased cell spread was visualized in both U373MG and U373MG-IE1 uninfected cells when RhoB was silenced (Fig. 2A and 2B).

In the context of HCMV infection, both U373MG and U373MG-IE1 RhoA and RhoC-depleted cells retained the basic feature observed in non-infected cells as described above, showing thin protrusions (Fig. 3A and 3B). Interestingly, in the HCMV infected RhoB-knockdown cells, an increased number of cellular projections were formed, a phenomenon which

Fig. 1. Rho isoform specific knockdown in U373MG and U373MG-IE1 cells. The expression of RhoA, RhoB and RhoC were determined in (A) U373MG cells and (B) U373MG-IE1 cells by Western Blot from whole protein extracts derived from each cell line after transduction with the indicated TRIPZ shRNA lentivirus vectors, induction with doxycycline and selection with puromycin. Actin served as loading control. The stable expression of IE1 in U373MG-IE1 cells was also confirmed. (C) U373MG cells, transduced with the lentivirus empty vector and induced with doxycycline, were infected with the recombinant HCMV AD169/IE2-EGFP virus at MOI=3 pfu/cell. Cells were fixed 8 hours after infection and nuclei were stained with DAPI. (bar: 10 µm).



has also been observed in primary HFF cells infected by HCMV [55]. Both HCMV infected glioblastoma cell lines with depleted each one of the three Rho isoforms were increased in volume, apparently due to the viral infection and the specific cytopathic effect HCMV causes.

The changes in the shape between the control cells and the three Rho proteins knockdown tested, both in uninfected and infected U373MG and U373MG-IE1 cells were measured using the Metamorph CellProfiler software. The analysis statistically further confirmed the alterations visualized by microscopy (Fig. 4).

Effect of HCMV on the proliferation rate of U373MG and U373MG-IE1 RhoA, RhoB and RhoC knockdown cells

We sought to determine the role of Rho GTPases in U373MG and U373MG-IE1 cells in uninfected and HCMV infected cells in terms of cell proliferation. Both cell lines were transduced with the appropriate shRNA lentivirus vectors knocking down RhoA, RhoB or RhoC, infected with HCMV AD169 when appropriate and subsequently tested in MTT assay. Depletion of either RhoB or RhoC in both the parental and the IE1 uninfected glioblastoma cells resulted in a significant inhibition of the proliferation rate compared to the control (empty vector) cells (Fig. 5A and 5B). Silencing of RhoA slowed down the cell growth of both parental and IE-1 derivative uninfected cells compared to the control cells but to a

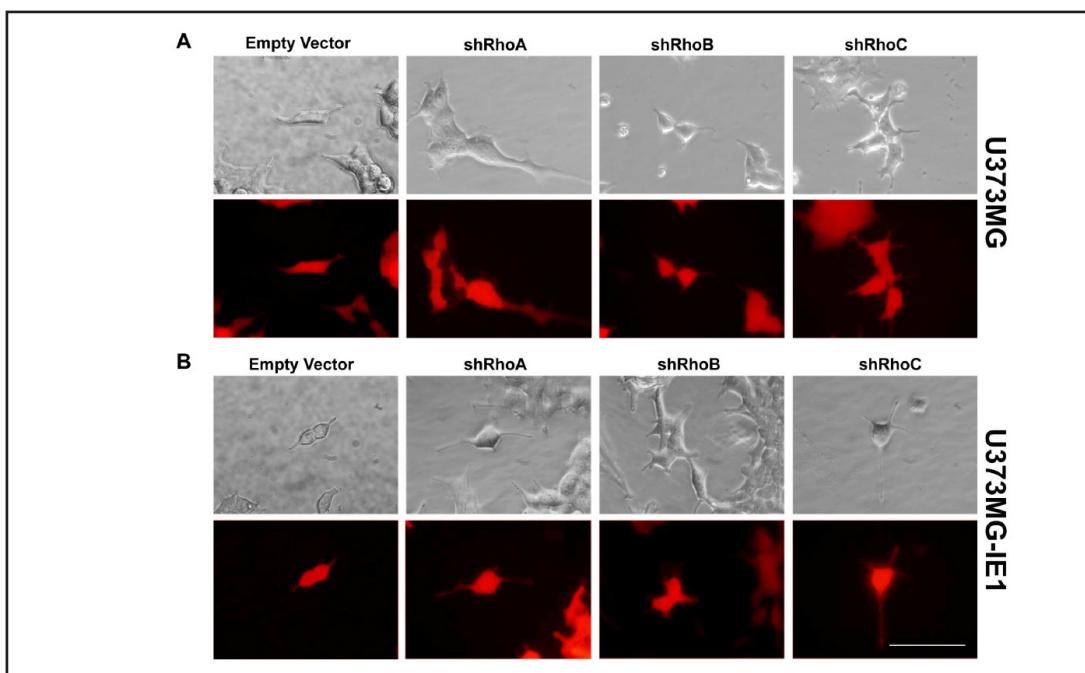


Fig. 2. Differential morphological changes in RhoA, RhoB and RhoC-depleted glioblastoma cells. U373MG and U373MG-IE1 cells were transduced with the indicated TRIPZ shRNA lentivirus vectors, induced with doxycycline and selected with puromycin. Doxycycline-treated TRIPZshRNA-expressing cells exhibit red fluorescence. Still images were obtained by timelapse microscopy 48h after plating (bar: 50 µm).

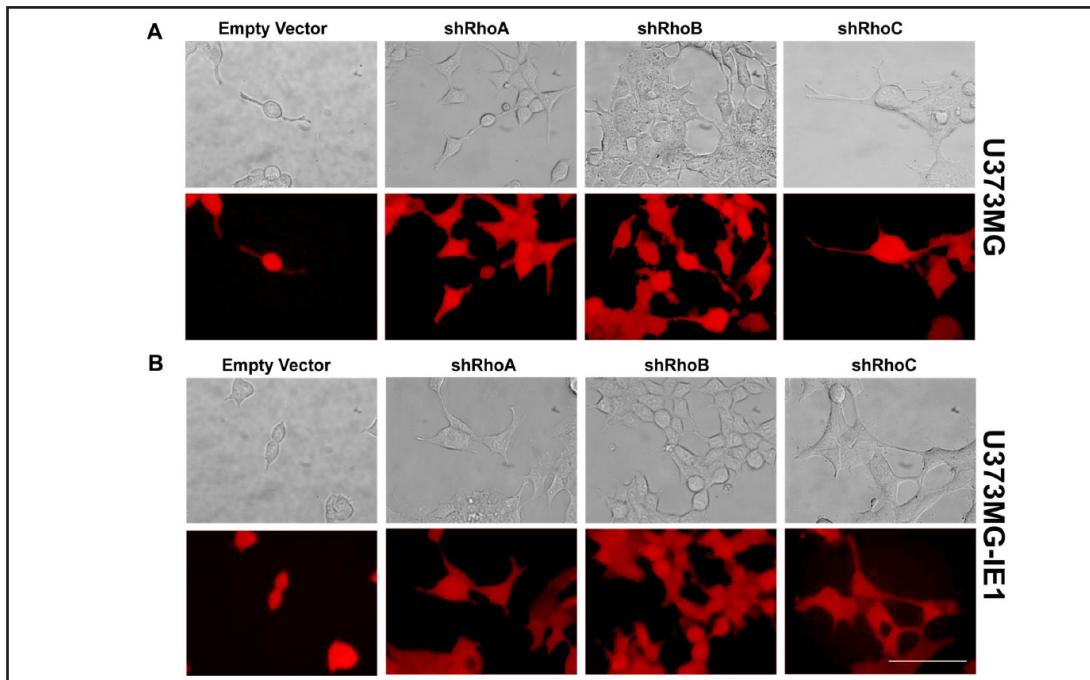


Fig. 3. Phenotype changes of RhoA, RhoB and RhoC-depleted HCMV infected glioblastoma cells. U373MG and U373MG-IE1 cells as in Fig. 2, were either mock infected or infected with HCMV AD169 (MOI=3 pfu/cell). Still images of live cells were captured 48h post infection (bar: 50 µm).

lesser extent compared to RhoB and RhoC silencing. HCMV infection alone did not affect the proliferation rate of either the U373MG or the U373MG-IE1 cells compared to the mock

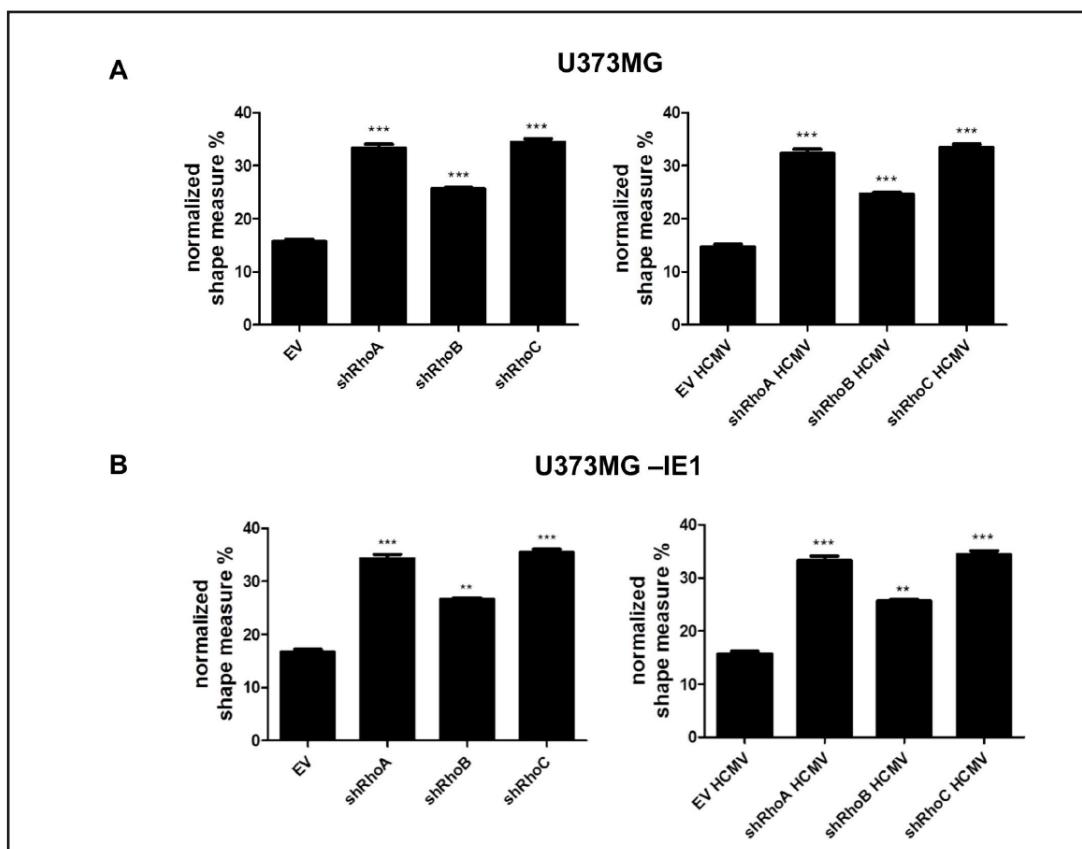


Fig. 4. Cell shape changes in RhoA, RhoB and RhoC knockdown uninfected and HCMV infected glioblastoma cells. U373MG and U373MG-IE1 cells were transduced with the appropriate TRIPZ shRNA lentivirus vectors, induced with doxycycline and selected with puromycin. HCMV AD169 at an MOI=3 pfu/cell was added in a subset of cells. Still images were captured 48 hours after infection, processed and analysed by Metamorph CellProfiler. The asterisks indicate statistical significance. ** indicate $P = 0.001$ to 0.01 , *** indicate $P = 0.0001$ to 0.001 . Data shown are means \pm SD of three independent experiments.

infected cells. Interestingly, HCMV infection of U373MG RhoA knockdown and U373MG-IE1 RhoA depleted cells did not cause a statistically significant difference compared to the control cells ($p = 0.1385$), whereas the proliferation state of the HCMV infected of RhoB or RhoC knockdown cells was inhibited ($p = 0.0941$ and $p = 0.0089$ respectively) (Fig. 5A and 5B).

Depletion of RhoA, RhoB or RhoC inhibits random migration

To characterize the function of RhoA, RhoB and RhoC in cell motility, we initially tested the migration of the corresponding free-moving Rho depleted U373MG and U373MG-IE1 cells (Supplementary movies: <http://www.med.uoc.gr/research-lab-clinical-virology.php>). Cells transduced with the empty vector often showed a long and polarized morphology whereas RhoA, RhoB and RhoC knockdown cells exhibited narrow lamellipodial structures at each projection and small dynamic protrusions along the elongated sides. When analyzing the route of individual cells from each Rho depleted cell type during 300 min migration period by timelapse microscopy, we found that the cells displayed a much shorter translocation than the control cells. Remarkably, the movement of RhoA silenced cells was dramatically limited, lacking their parental polarization and rather presenting an amoeboid fashion with cycles of expansion and contraction of the cell body. Infection with HCMV of the same cells did not cause significant changes in their motility and they rather showed reduced random migration speed. On the contrary, HCMV infected U373MG-IE1 cells exhibited a higher

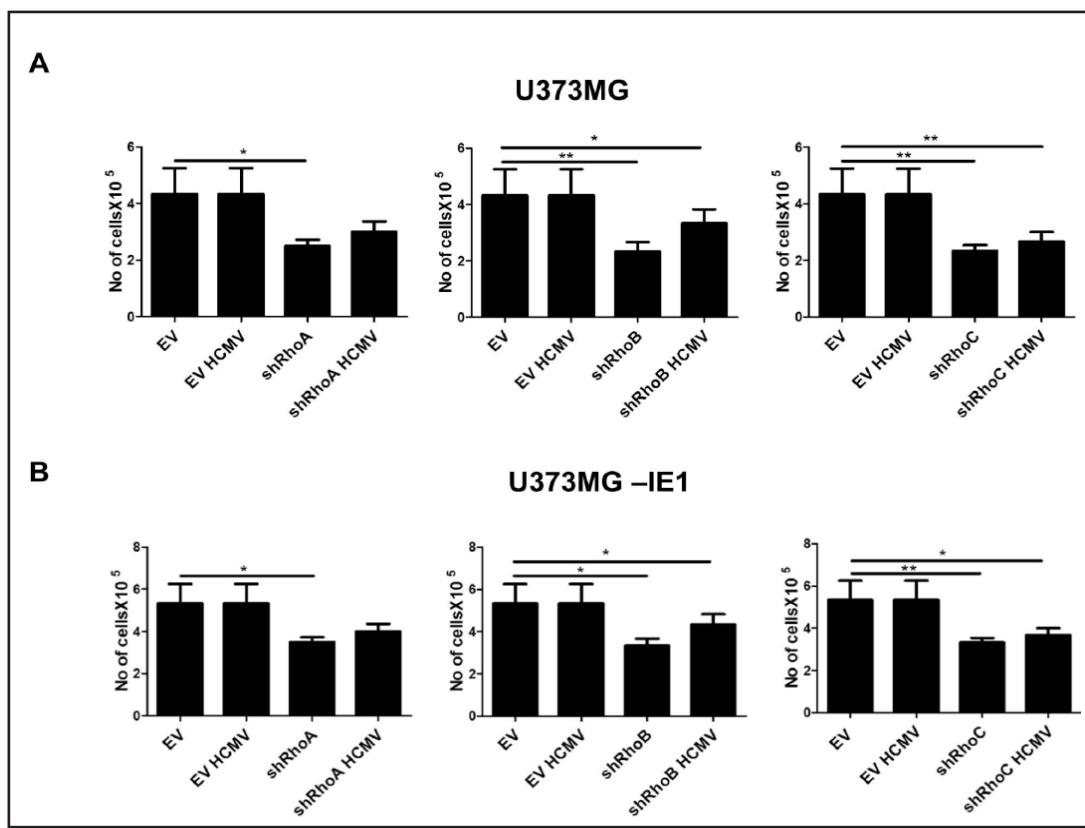


Fig. 5. Proliferation rate of RhoA, RhoB and RhoC knockdown uninfected and HCMV infected glioblastoma cells. The cells were transduced as in Fig. 2 and subsequently were either mock infected or infected with HCMV at MOI=3 pfu/cell and 3 days after infection the cell proliferation rate was measured employing MTT Assay. The asterisks indicate statistical significance. * indicate $P = 0.01$ to 0.1 , ** indicate $P = 0.001$ to 0.01 . Data shown are means \pm SD.

motility speed, enhancing the defect in the migration caused by the knockdown of each Rho protein and showing longer paths. Even in this more favorable context for the IE1-expressing glioblastoma cells, the free-movement of shRhoA cells was the most severely impaired compared to the control cells.

Rho GTPases are required for glioblastoma cell migration

The role of RhoA, RhoB and RhoC in cell migration was explored in uninfected and HCMV infected parental U373MG and their derivative cells expressing the HCMV IE1 protein. Results showed that the knockdown of each of the three Rho isoforms significantly decreased the average migration rate of U373MG cells compared to empty vector cells, as determined by wound healing assays. Moreover a differential reduction in the speed of cell movement was monitored, depending on which Rho protein was depleted. Among all three Rho proteins tested, the healing rate was considerably faster in RhoC knockdown cells, moderately slower in RhoB depleted cells while strikingly, the movement of RhoA silenced cells was significantly limited and they appeared almost stuck throughout the course of wound healing assay (Fig. 6A). The aforementioned observations in Rho depleted cells did not change upon infection of the cells with HCMV (Fig. 6B). The migration rate of U373MG-IE1 cells was also decreased in RhoA, RhoB and RhoC knockdown cells compared to the empty vector cells. However, this difference was remarkable compared to the parental cells and apparently, the expression of IE1 protein accelerates cell migration compared to the non-IE1 expressing cells (DATA NOT SHOWN). In contrast to Rho depleted parental glioblastoma cells, HCMV infection of the U373MG-IE1 cells after knockdown of RhoA, RhoB or RhoC restored the migratory behavior

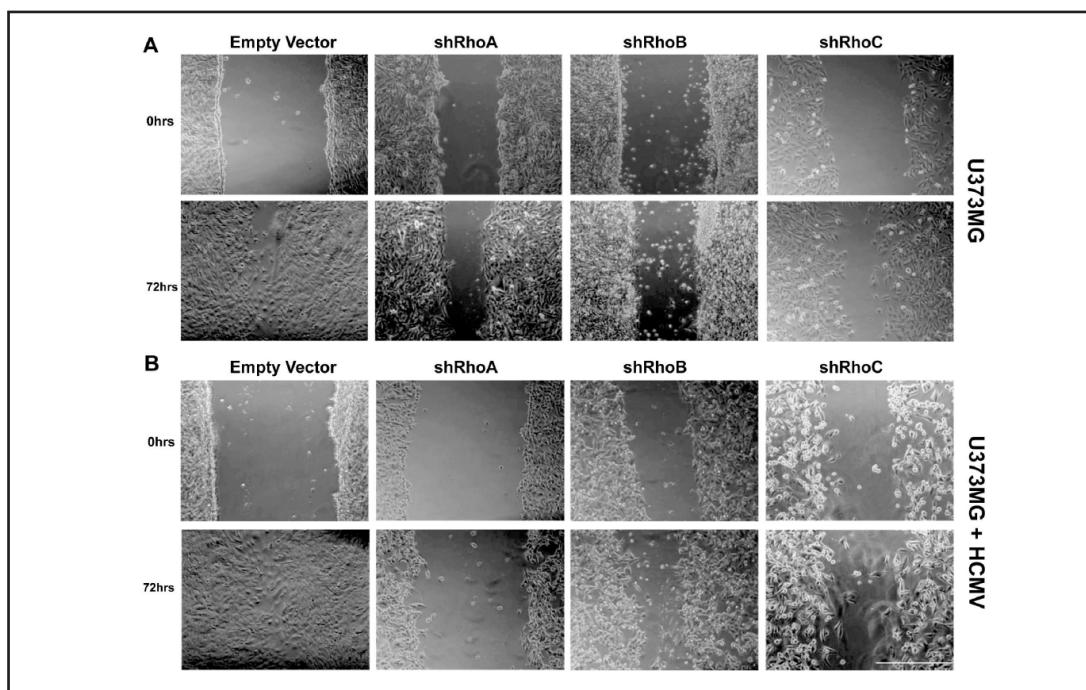


Fig. 6. Effect of RhoA, RhoB and RhoC depletion on wound healing migration in uninfected and HCMV infected U373MG cells. U373MG were plated in 6-well plates, treated as in Fig. 2 and were either HCMV infected (MOI=3 pfu/cell) or not. The monolayer was “wounded” 48 hours post infection with a 10 µl sterile pipette tip and the detached cells were removed. Images were taken at 0, 3, 6, 9, 12, 24, 48 and 72 h after the scratch to monitor cell motility by timelapse microscopy. The initial (0 h) and final (72 h) time points of the assay are presented. (bar: 50 µm).

of the cells at comparable levels to the empty vector cells, either in the presence or absence of viral infection.

The area of the wounds was also recorded at different time points (3, 6, 9, 12, 24, 48, 72 hours) following the generation of the wounds. A marked delay in wound closure was observed in RhoA, RhoB or RhoC knockdown U373MG cells compared to the empty vector cells (Fig. 7A). Calculations on the area of the wounds demonstrated that in control cells, only $53\% \pm 1.9\%$ of the initial wound area was left as opposed to all three Rho depleted cells where the wounds areas did not change significantly, as only $80\% \pm 1.1\%$ of them were covered. Close examination of cells at the wound edge revealed that control cells displayed a polarized phenotype, with cell protrusions perpendicular to the wound along with rapid elongation of cell projections. In contrast, cells with depleted Rho proteins exhibited a less evident polarized phenotype, with RhoA knockdown manifesting a more drastic disruption of polarity, followed by RhoB and RhoC silencing. Although these cells could still form protrusions at the wound edge, their directions were more random and a substantial proportion of cells displayed multiple short protrusions or protrusions parallel to the wound.

These phenotypes between the Rho depleted cells and the control cells remained unaltered during the course of HCMV infection. As regards the U373MG-IE1 cells, the defective closure capacity compared to the empty vector cells was also true when RhoA, RhoB or RhoC were silenced whereas HCMV infection of the same cells resulted in a remarkable increase in the closure efficiency of the wounds (Fig. 7B). These data collectively indicate that RhoA, RhoB and RhoC play a favorable role in cell migration of glioblastoma overexpressing IE1 protein cells and also that HCMV confers a migration advantage to these cells in Rho proteins depleted cells.

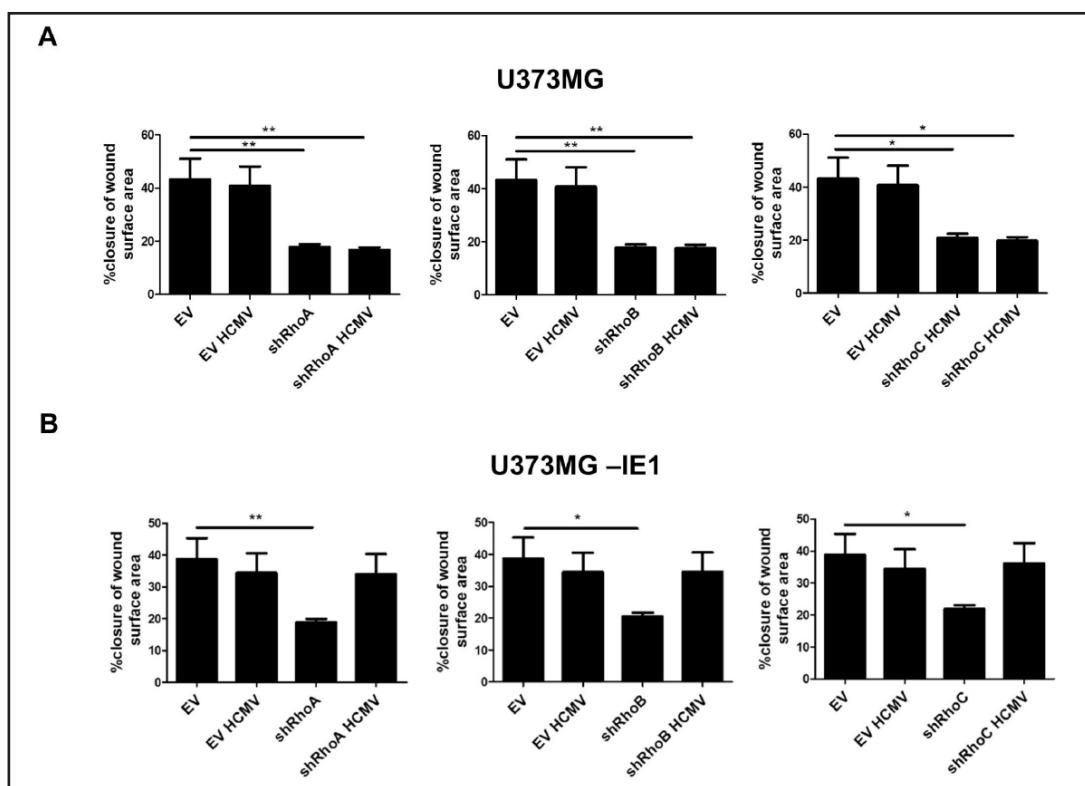


Fig. 7. Quantification of wound healing assay in RhoA, RhoB and RhoC knockdown U373MG and U373MG-IE1 cells. Motility was quantified by measuring the decrease in the denuded area at 0 hours and 48 hours and presented as the average decrease in the number of pixels with standard deviation in three independent experiments. The asterisks indicate statistical significance. * indicate $P = 0.01$ to 0.1 , ** indicate $P = 0.001$ to 0.01 . Data shown are means \pm SD of experiments in triplicate.

Discussion

In the current study, we demonstrate, for the first time, an implication of Rho GTPases in cell morphology, proliferation and migration in human cytomegalovirus infected glioblastoma cells. Foremost, we observed that knockdown of RhoA resulted in an elongated cell shape with thin protrusions in non-infected and HCMV infected U373MG as well as in their derivative U373MG-IE1 cells. Likewise, depletion of RhoC led the cells to obtain an elongated, mesenchymal-like shape. The above observations are consistent with earlier studies depleting the same Rho isoforms both in prostate and breast cancer cells [59, 62]. The overexpression of HCMV IE1 protein alone did not bring about any additional effect regarding the phenotype of the Rho knockdown cells, thus excluding any direct modulatory role of IE1 on cell morphology. The morphological changes induced in the glioblastoma cells after RhoA and RhoC depletion remained evident, even in the context of HCMV infection, despite the cytopathic effect caused by the virus, highlighting an imposing role Rho GTPases on cell shape. Although Rho GTPases have been shown to enhance malignant transformation and proliferation rate, RhoB is rather assumed as a negative regulator of these processes [63, 64]. However, recent data have shown that RhoB expression is induced under conditions such as DNA damage, or treatment with growth factors or cytokines, suggesting that under particular circumstances, RhoB may favor towards human malignancy, including glioblastoma tumors [65, 66]. In the present study, we show that when RhoB is silenced, both parental and their derivative-IE1 cells show a reduction in spread area compared to the RhoA or RhoC knockdown cells, an effect which has attributed to subsequent reduction of total surface levels of b1 integrin [67, 68]. Moreover, in the presence of HCMV, an increased

number of cellular projections was formed, a phenomenon which has also been observed in HCMV infected primary HFF cells, particularly at the late stages of lytic infection [49]. These findings further corroborate the idea of a key role for RhoB in the regulation of cell shape.

Regarding to the proliferation rate of uninfected parental and IE1-expressing glioblastoma cells, the deficiency of RhoB or RhoC resulted in a significant inhibition of their proliferation state while RhoA deficiency reduced the cell growth of both U373MG and U373MG-IE1 cells to a lower degree. This difference reflects the evidence that RhoA regulates actin polymerization, cell adhesion and myosin activity [7, 69] whereas RhoB and RhoC control cell survival and cell proliferation, respectively [70]. This concept is furthermore supported by our observation that HCMV infection of U373MG and U373MG-IE1 RhoA depleted cells does not seem to affect significantly their proliferation rate, whereas the proliferation state of the HCMV infected of both parental and U373MG-IE1 RhoB or RhoC knockdown cells is statistically significantly inhibited. HCMV infection has been involved in human tumors influencing cell properties such as migration, invasion or cell signaling [31, 32, 71]. The results exploring the Rho GTPases silencing in association to cell proliferation of these cells and in the context of HCMV infection, indicate that RhoB and RhoC depletion in U373MG glioblastoma cells rather results in the inhibition of the oncomodulatory effects of the virus. Interestingly, HCMV infection partially restored the growth rate of the RhoA knockdown glioblastoma cells, suggesting that the virus can differentially interfere with the proliferation state of the cells, depending on which Rho GTPase is depleted. This was also the case in the U373MG-IE1 RhoA knockdown cells. Apparently, the expression of IE1 alone and despite the oncogenic properties this protein may possess, it was not sufficient to confer a proliferation advantage to these cells and the active viral gene expression is required.

The alterations in the free movement of Rho knockdown cells were also monitored. The cells display diminished movement with U373MG and U373MG-IE1 depleted RhoA, RhoB or RhoC cells lacking their polarization and rather presenting an amoeboid formation with cycles of expansion and contraction of the cell body reinforcing the evidence that Rho GTPases contribute to different features of cancer, such as invasion and metastasis [72]. Infection with HCMV does not change significantly their motility and they rather show reduced random migration speed. In contrast, HCMV infected U373MG-IE1 cells exhibit a higher motility rate, enhancing the defect in the migration caused by the deficiency of each Rho protein. It is worth noting the fact that even in the absence or presence of HCMV, parental and their derivative IE1-knockdown RhoA cells exhibited the lowest motility rate even in this more favorable for the IE1-expressing glioblastoma cells context, providing an additional proof reinforcing the significant role of RhoA to promote cell migration [73, 74].

The aforementioned observations prompted us to investigate moreover the average migration of non-infected and infected U373MG depleted one of the three Rho isoforms cells, with RhoB and RhoC knockdown cells having the faster healing rate and in accordance with studies highlighting the role of these GTPases mainly in malignant transformation and to a less extend in motility [75], whereas the movement of RhoA silenced cells is decreased (Fig. 6A and 6B), revealing once more the differential role of RhoA, RhoB and RhoC in migration and invasion [76-78]. Interestingly, even if the migration rate of non-infected U373MG-IE1 depleted RhoA, RhoB and RhoC cells was also decreased, however, in the presence of HCMV, these cells partially restored their increased motility rate in migratory state, in contrast to the Rho depleted parental glioblastoma cells. This acceleration in cell migration could be due to the expression of viral genes, including IE1 protein since earlier studies have demonstrated that the IE1 protein suppresses apoptosis and facilitates oncogenesis [79, 80] and more recent studies that positively contributes to the replication cycle of HCMV [81, 82].

Taken together, our data suggest an important implication of RhoA, RhoB and RhoC GTPases in morphology, proliferation and motility of both uninfected and HCMV infected U373MG glioblastoma cells. HCMV has been shown to manage signaling networks in infected cells, including the activation of G proteins [83] in order to reserve infection and viral spread. In this study, we display that downregulation of RhoA, RhoB and RhoC proteins in infected glioblastoma cells decreases their proliferation rate and their migration status and inhibits

HCMV from worsening the malignant glioma pathogenesis. Interestingly, the presence of the HCMV Immediate-Early protein IE1 facilitates HCMV to retain its oncomodulatory advantage in these RhoA, RhoB and RhoC depleted cells [84]. Therefore, we suggest a possible direct or indirect involvement of Rho small GTPases during HCMV infection with potential role in human glioblastoma cells.

Acknowledgements

We thank S. Kim (Seoul, Korea) for kindly providing the U373MG and the U373MG-IE1 cells.

This project was funded by The National Plan for Science, Technology, and Innovation (MAARIFAH), King Abdulaziz City for Science and Technology, Kingdom of Saudi Arabia (Award number 12-MED2652-02).

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interest and nothing to disclosure.

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