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ORIGINAL ARTICLE

Chlorogenic Acid in *Viscum album* Callus is a Potential Anticancer Agent against C6 Glioma Cells

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ABSTRACT

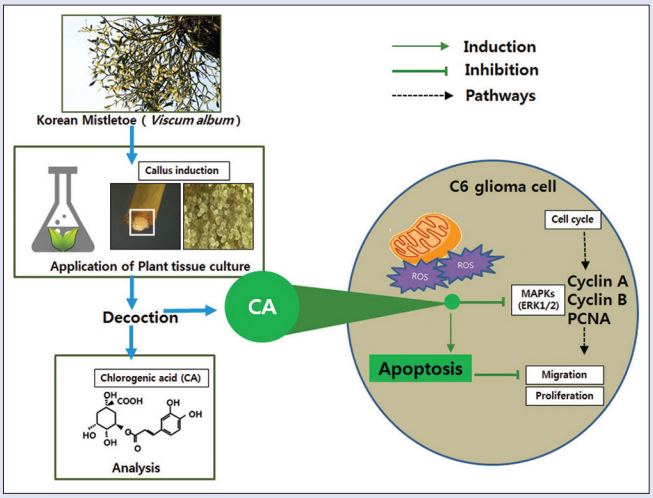
Background: Chlorogenic acid (CA), a polyphenolic component of fruits, vegetables, coffee, wine, and olive oil, has beneficial effects on human heath, including antioxidant and anticancer effects. However, its precise effects on glioma have not been examined. **Objective:** Our study aimed to explore the anticancer effects of CA obtained from *Viscum album* callus on C6 glioma cell migration and proliferation. **Materials and Methods:** Anticancer potency was analyzed by the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt assay to assess the ability to inhibit cell growth and proliferation. Cell mobility was investigated based on the Boyden chamber and the scratch wound healing assay. Factors involved in cell cycle progression were evaluated by mRNA and protein expression. Cell death was determined by staining with specific dyes and fluorescence microscopy. **Results:** CA significantly reduced C6 glioma cell proliferation and migration. Furthermore, it induced reactive oxygen species generation and apoptotic cell death. Treatment with CA also suppressed extracellular signal-regulated kinase ½ (ERK½) phosphorylation and the gene expression of cyclins E and A. **Conclusion:** Our results show that CA may regulate glioma cell migration and proliferation via modulation of ERK½ phosphorylation and cell cycle regulation. Thus, it might be a potent anticancer agent in preventing progression of glioma.

Key words: Apoptosis, chlorogenic acid, extracellular signal-regulated kinase ½, glioma, proliferation

SUMMARY

- *Viscum album* callus contains a potential anticancer agent, chlorogenic acid (CA) in against C6 glioma cells. CA can induce apoptosis in C6 glioma cells by reactive oxygen species generation. CA regulates p-extracellular signal-regulated kinase ½ and cell cycle in C6 glioma cells.

Abbreviations used: CA: Chlorogenic acid; ERK½: Extracellular signal-regulated kinase ½; CDKs: Cyclin-dependent kinases; MAPK: Mitogen-activated protein kinase; PCNA: Proliferating cell nuclear antigen.



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INTRODUCTION

Glioma is a type of malignant tumor originating from glial cells in the brain or spinal cord.[1] Until recently, glioma was classified as malignant regardless of histologic diagnosis.[2] In particular, malignant brain glioma causes cell proliferation and rapid invasion of surrounding tissues and is difficult to treat with conventional therapies.[3,4] Therefore, the disease is characterized by poor prognosis despite chemotherapy and surgery.

The cell cycle is divided into two phases, namely chromosome division (the mitosis [M] phase) and preparation for cell duplication (the synthesis [S] phase). The Gap 1 (G1) and Gap 2 (G2) phases are preparatory to the S and M phases, respectively. During cell cycle progression, G1, S, G2, and M phases are periodically repeated under the control of a system of cooperating cyclins and cyclin-dependent kinases (CDKs).[5,6] The expression of CDK, cyclin E, and cyclin A is

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precisely regulated by multiple protein kinases and ubiquitin-dependent proteases, and abnormal functioning of these regulatory proteins plays a crucial role in cancer cell proliferation.[7] In particular, diverse studies have reported that the malignant behavior of glioma, involving abnormal migration and proliferation, is related to the activation of mitogen-activated protein kinase.[8-10] Therefore, chemotherapeutics targeting extracellular signal-regulated kinase ½ (ERK½) phosphorylation, which interfere with cell cycle progression and cell motility, are strongly required.

Viscum album L. (mistletoe) is an oriental medicinal herb that is widely used for the prescription of several diseases, such as cancer, hepatitis, and skin diseases.[11,12] However, the anticancer properties of callus induced from mistletoe has not yet been explored. Chlorogenic acid (CA), a polyphenolic component, is a complex of quinic and caffeic acids and functions as an antioxidant and anticancer agent.[13] Fruits, vegetables, coffee, wine, and olive oil, all containing abundant CA, show advantageous effects on human health.[14] However, the anticancer effect of CA on glioma has been poorly studied thus far. Thus, this study aimed to investigate the impact of CA, which we found to be contained in the active substance of mistletoe callus, on glioma cell migration and proliferation and provide a basis for the development of possible therapeutic strategies that can inhibit the spread of glioma.

MATERIALS AND METHODS

Reagents and plastic ware

The Murashige and Skoog culture medium for plant cells was purchased from Merck (Darmstadt, Germany). The plastics and Dulbecco's Modified Eagle's Medium (DMEM) for cell culture were purchased from Thermo Fisher (Waltham, Massachusetts, USA). WelCount™ Cell proliferation assay kit was purchased from Welgene (Gyeongsangbuk-Do, Korea). The Diff-Quick stain was obtained from Polysciences (Warrington, Pennsylvania, USA), while 2',7'-dichlorodihydrofluorescein diacetate (H_2DFC-DA), Hoechst solution, and ethidium homodimer (EthD-1) were purchased from Thermo Fisher. Antiphosphorylated ERK½ antibody, anti-total (T)-ERK½ antibody, and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody were obtained from cell signaling technology (Beverly, Massachusetts, USA). The primers were purchased from Bioneer Corporation (Daejeon, Korea). CA and other chemicals were purchased from Merck (Darmstadt, Germany).

Induction of *Viscum album* callus and preparation of ethanolic extracts

The stem of the plant was used for callus induction of *V. album* according to a previously described method.[15] Briefly, the sterilized explants of *V. album* stem (1 cm) were incubated under aseptic conditions on Murashige and Skoog medium (1.0%) supplemented with casein (0.001%), sucrose (3%), kinetin (0.000001%), and plant agar (0.6%) at darkroom (25°C ± 1°C). After 30 days, the callus formation indicated from the edge of stem explants of *V. album*. The extract of *V. album* callus was prepared according to a slightly modified previously described method.[16] Briefly, the callus form and explants (200 g) were finely grinded, and then, the powder was extracted for 24 h using 1000 mL of ethanol. The precipitate was filtered from ethanol extraction, and then, only supernatant was concentrated by evaporation at 60°C in vacuum. Moreover, the precipitate from which ethanol was completely removed through evaporation was dissolved in 50 mL of sterile deionized water (SDW). The water-soluble extract was lyophilized using the freeze-dryer at −60°C.

Measurement of chlorogenic acid

The quantity of CA in *V. album* calli was performed by the high-performance liquid chromatography analysis (HPLC) using C_{18} column (Sunfire C_{18} ODS 4.6 mm × 150 mm column) connected to a photodiode array detector (Waters Corporation, USA). The mobile phase was used to a mixture of 0.1% acetonitrile and 0.1% formic acid in water at a flow rate of 1 mL/min. CA (5 mg/mL in SDW) standard solution was filtered through a membrane filter (0.45-μm), after which HPLC was performed.

Cell culture and cell viability assay

Rattus norvegicus brain glioma (C6 glioma) cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured in DMEM containing fetal bovine serum (FBS) and penicillin-streptomycin solution (1%) at 37°C in a 5% CO_2 atmosphere. C6 glioma cells (5×10^4 cells/mL) were seeded and incubated in 96-well microplates for 24 h, and then, the culture medium was replaced with serum-deficient DMEM. After 24 h, the medium was replaced with diverse concentrations of CA (10, 30, 100, and 300 μM) for 24 h. The solution of WelCount™ Cell proliferation assay kit (10 μL/each well) was added and further incubated for 1 h, following which cell viability was determined as relative wavelength (absorbance at 450 nm) with the untreated group.

Chemotaxis assays

To determine the anticancer effect of CA, the test of anticell migration performed the two types of chemotaxis assays using the scratch wound healing (WH) assay and the Boyden chamber (BC) assay. First, WH assay was performed as previously described.[16] Briefly, C6 glioma cells were seeded in 6-well plates (1×10^5 cells/mL) and cultured for 24 h, and then, the culture medium replaced with serum-free DMEM further incubated 24 h. The center of culture dish was to scratch with a sterile 200-μL pipette tip. Moreover, the medium was replaced serum-deficient DMEM and treated with CA (100 and 300 μM) for 24 h. Cell-migrated images were recorded using an IX71 microscope (Olympus; Tokyo, Japan) for 24 h. The migration rates were determined based on the percentage of migration area at 0 and 24 h using the ImageJ software image J software (NIH, Bethesda, MD, USA). Next, BC assay was performed as previously described.[17] The grown cells were trypsinized and harvested in DMEM containing 0.1% bovine serum albumin (BSA). The cell concentration was adjusted at 1×10^6 cells/mL in DMEM containing 0.1% BSA. The lower chamber was loaded with medium with or without serum and CA (100 and 300 μM). A membrane coated with Type I collagen was placed on the low chamber. After combining the upper chamber with the lower chamber, the upper chamber was filled with 50 μL of a C6 glioma cell suspension. And then, the BC was incubated at 37°C in a 5% CO_2 atmosphere. After incubation for 90 min, nonmigrating cells were removed on membrane according to the manufacturer's instructions (Neuro Probe; Maryland, USA). The membranes were stained with the Diff-Quick kit. The migrated cells were analyzed using the ImageJ software.

2',7'-dichlorodihydrofluorescein diacetate staining

C6 glioma cells (1×10^4 cells/mL) were seeded in an 8-well chamber for 24 h and then incubated with serum-deficient medium for 24 h. Next, C6 glioma cells were incubated in the DMEM and treated with CA (100 and 300 μM) for 24 h. And then, cultured medium replaces in phosphate-buffered saline (PBS). The cells were incubated with H_2DFC-DA (10 μM in PBS) solution for 20 min. The fluorescence of H_2DFC-DA staining was confirmed using the fluorescence microscopy (excitation 492 nm and emission 527 nm) (K1-fluo, Nanoscope system, Daejeon, Korea). Fluorescence intensity was measured using the ImageJ software.

Cell death assay

Cell death assays were performed as previously reported.[18] Cell death was analyzed by staining with Hoechst and EthD-1. First, the C6 cells (1×10^4 cells/mL) were treated with the absence or presence of CA (100 and 300 μM) for 24 h and then fixed with paraformaldehyde for 15 min at 25°C. The staining was performed with Hoechst (167 μM) at 37°C for 20 min, and then, the fluorescence of cells was measured using the fluorescence microscopy (excitation 361 nm and emission 497 nm). In the case of EthD-1 staining, the fixed cells were incubated with EthD-1 (0.5 μM) in PBS for 30 min at 25°C. Stained samples were observed by fluorescence microscopy (excitation 528 nm and emission 617 nm), and signal intensity was measured using the ImageJ software.

Immunoblot

Immunoblot assay was performed as previously reported.[19-20] Briefly, cell lysates quantified in equal amounts (20 μg) and then separated by electrophoresis in 12% sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes at 4°C for 2 h. The PVDF membranes were replaced with 5% BSA solution for 1 h at 25°C and then incubated with the 1:500 dilution of antibodies such as anti-p-ERK½, anti-ERK½ and anti-GAPDH for 18 h at 4°C. After washing with Tris-buffered saline-Tween 20, the PVDF membranes were incubated with a 1:500 dilution of the secondary antibody for 1 h. The protein expression levels were detected by chemiluminescence and analyzed using ImageJ software.

RNA levels expression assay

RNA levels expression assays were performed as previously reported.[21] Total RNA extraction was performed using the TRI reagent according to the manufacturer's instructions. Next, cDNA synthesis was performed using the Superscript III First Strand cDNA Synthesis Kit according to the manufacturer's instructions. Real-time polymerase chain reaction (RT-PCR) assay was conducted using an Ab7500 RT-PCR detection system with power SYBR Green PCR mix under the following condition: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The following primers were used for RT-PCR: cyclin E, forward primer, 5'-ATG TCC AAG TGG CCT ACG TC-3' and reverse primer, 5'-TCT GCA TCA ACT CCA ACG AG-3'; cyclin A, forward primer, 5'-GCT TTT AGT GCC GCT GTC TC-3' and reverse primer, 5'-AGT GAT GTC TGG CTG CCT CT-3'; proliferating cell nuclear antigen (PCNA), forward primer, 5'-TCA CAA AAG CCA CTC CAC TG-3' and reverse primer, 5'-CAT CTC AGA AGC GAT CGT CA-3'; and GAPDH, forward primer, 5'-TGG AGT CTA CTG GCG TCT T-3' and reverse primer, 5'-TGT CAT ATT TCT CGT GGT TCA-3'.

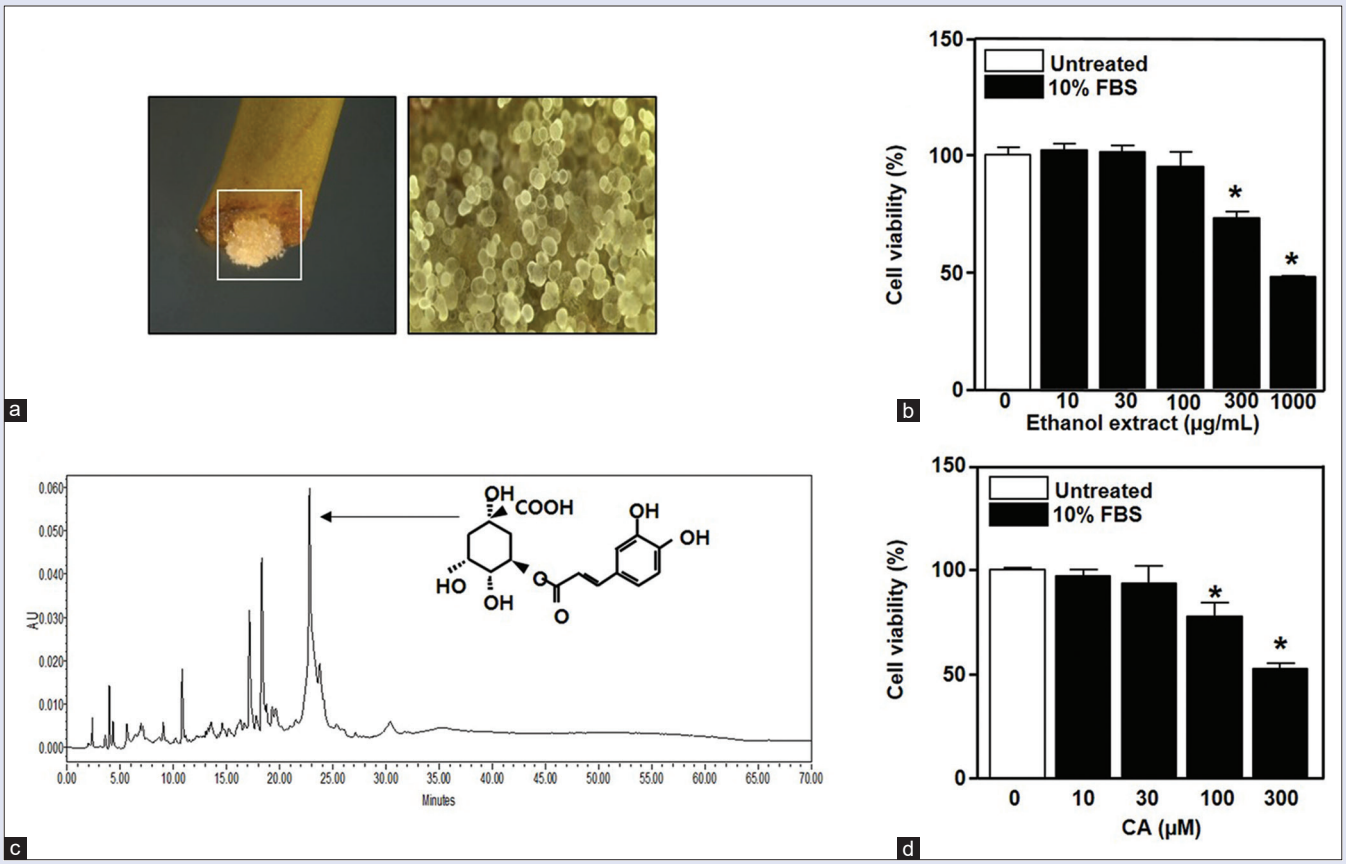

Figure 1: Effect of chlorogenic acid and *Viscum album* callus ethanolic extract on C6 glioma cell viability. (a) Callus induction from stem explant of *Viscum album*. (b) C6 glioma cells were treated with ethanol extract (10, 30, 100, 300, and 1000 μg/mL) for 24 h. Cell viability was tested using the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt assay. (c) Liquid chromatography–mass spectrometry spectrum of ethanol extract and the chemical structure of chlorogenic acid. (d) C6 glioma cells were treated with chlorogenic acid (10, 30, 100, and 300 μM) for 24 h. Cell viability was tested using the XTT assay. The cell viability in the fetal bovine serum-only treated group was considered to be 100%. The data are presented as mean ± standard deviation ($n = 3$). *$P < 0.05$ compared with untreated cells. CA: Chlorogenic acid; FBS: Fetal bovine serum

Statistical analysis

Data were evaluated using GraphPad Prism 5.0 (GraphPad Inc., La Jolla, CA, USA). The results are expressed as the mean ± standard deviation (SD) of at least three independent experiments ($n \geq 3$). Differences among the means were assessed using a one-way analysis of variance followed by Tukey's multiple range tests. The statistical significance was set at $P < 0.05$.

RESULTS

Ethanolic extract of *Viscum album* callus (EV) and chlorogenic acid suppressed C6 glioma cell viability

Calli were formed after 15 days [Figure 1a]. In the HPLC assay, CA was recognized among the mass components of the calli by its retention time and consistent molecular weight of 354.31 [Figure 1c]. As shown in Figure 1b and d, EV reduced cell viability by 50% at 1000 μg/mL, while CA reduced the viability by 50% at 300 μM.

Chlorogenic acid reduced C6 glioma cell migration

To test the inhibitory effect of CA on the migration of C6 glioma cells, we employed the BC assay and the WH assays. As shown in Figure 2a and b, a 90-min incubation with 10% FBS stimulated the migration of C6 glioma cells by 300%, relative to that of quiescent cells. Cell migration was notably suppressed by 300 μM CA. This inhibitory effect of CA on cell migration was confirmed using the WH assay, wherein CA treatment significantly reduced the percentage of migration area [Figure 2c and d].

Chlorogenic acid-induced C6 glioma cell death through the action of reactive oxygen species

Reactive oxygen species (ROS)-induced apoptosis is critical in anticancer therapy.[22] To confirm whether CA can induce apoptosis in C6 glioma cells by ROS generation, we performed cell staining with H_2DCF-DA, Hoechst, and EthD-1. As shown in Figure 3, CA substantially induced ROS generation (indicated in green in the H_2DCF-DA staining panels), nucleus fragmentation (indicated in sky

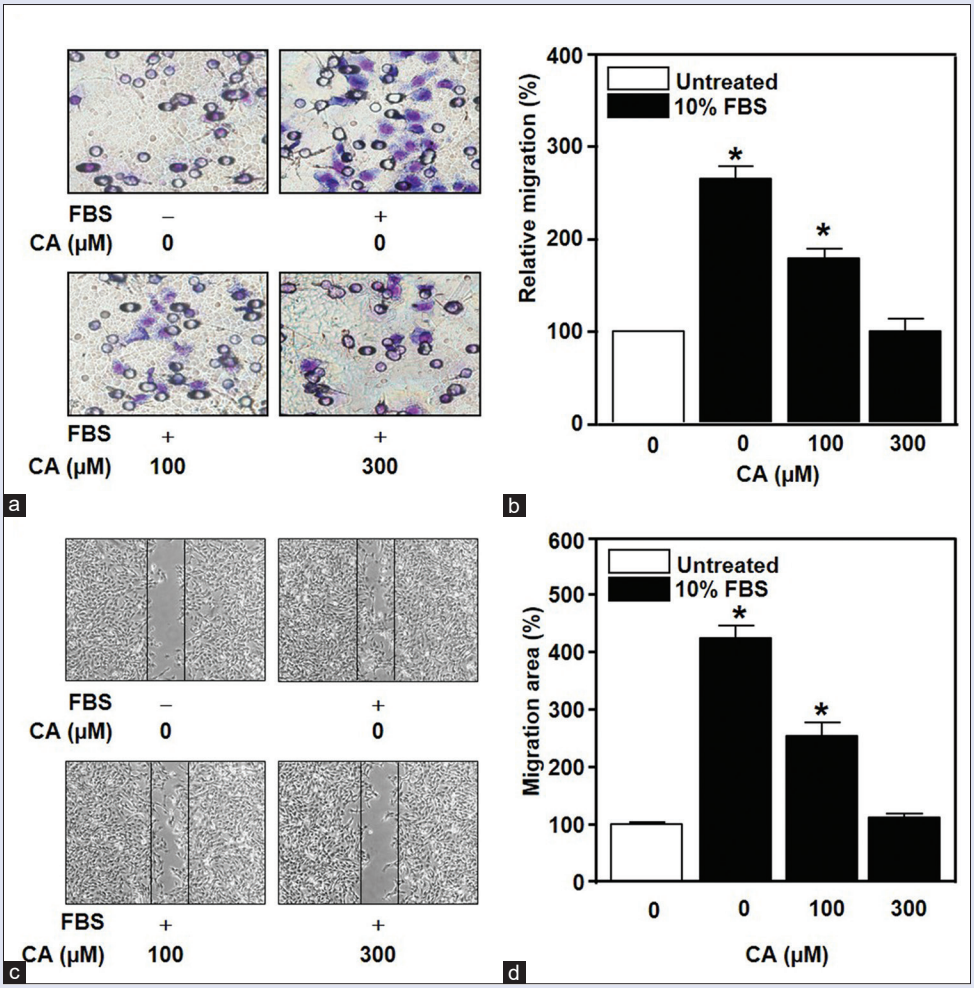

Figure 2: Effect of chlorogenic acid on the migration of fetal bovine serum-stimulated C6 glioma cells. (a) The photographs indicate the migrated cells in the presence or absence of chlorogenic acid (100 and 300 μM) and 10% fetal bovine serum for 90 min. (b) The bar graphs were obtained from panel A. The migration of untreated cells was considered to be 100%. (c) Confluent C6 glioma cell cultures were scratched using a sterilized 200-μL pipette tip and then the cells were cultured in the presence or absence of CA (100 and 300 μM) and 10% fetal bovine serum for 24 h. The photographs indicate the migrated C6 glioma cells in the wound area. The black bold lines indicate distance at time 0. (d) The bar graphs were obtained from panel C. The migration area in untreated cells was considered to be 100%. The data are presented as mean ± standard deviation ($n = 3$). *$P < 0.05$ compared with untreated cells. CA: Chlorogenic acid; FBS: Fetal bovine serum

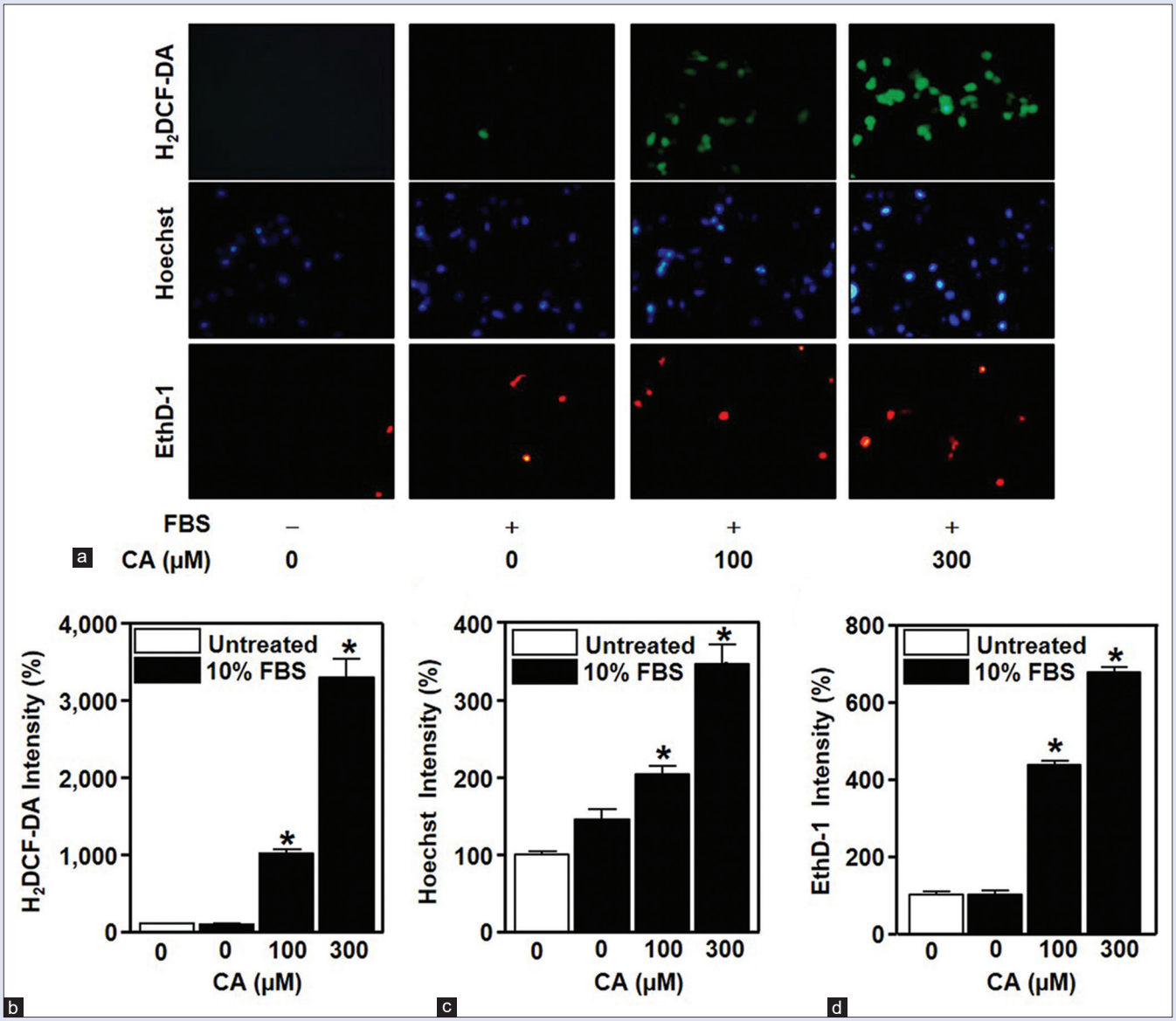

Figure 3: Effect of chlorogenic acid on C6 glioma cell death. C6 glioma cells were seeded in 8-well chambers and treated with CA (100 and 300 μM) for 24 h. (a) The cells were stained with 2′,7′-dichlorodihydrofluorescein diacetate, Hoechst, and ethidium homodimer. The images were obtained using a fluorescence microscope. (b-d) Bar graphs obtained from the images in panel A, based on the intensity of 2′,7′-dichlorodihydrofluorescein diacetate, Hoechst, and ethidium homodimer staining, respectively. The intensity obtained in control cells was considered to be 100%. The data are presented as mean ± standard deviation (n = 3). *P < 0.05 compared with untreated cells. CA: Chlorogenic acid; FBS: Fetal bovine serum; H_2DCF-DA: 2′,7′-dichlorodihydrofluorescein diacetate; EthD-1: Ethidium homodimer

blue in the Hoechst staining panels), and cell death (indicated in red in the EthD-1 staining panels).

Chlorogenic acid modulated the expression of phosphorylated extracellular signal-regulated kinase ½ and cell cycle regulators

Several studies have reported that phosphorylated ERK½ induces the proliferation of diverse types of cells.[16,23] We tested whether CA affected the expression of cell cycle regulators, as well as ERK½ phosphorylation, in C6 glioma cells via immunoblotting analysis. As shown in Figure 4a and 4b, cell exposure to FBS resulted in a nearly 100% increase in ERK½ phosphorylation. Notably, 300 μM CA completely inhibited FBS-induced ERK½ activation. It is known that cell cycle progression is influenced by a system of cyclins and CDKs.[24] Thus, to determine whether CA could influence the cell cycle, we examined the mRNA expression of cell cycle markers such as cyclin A, cyclin E, and PCNA [Figure 4c, d and e]. CA (300 μM) significantly suppressed the expression of cyclin A and cyclin E (P < 0.05).

DISCUSSION

Although chemotherapy is considered as the standard treatment for tumors, systemic administration of chemotherapeutics may not result in sufficient therapeutic action at the tumor site and may produce side effects.[25] Therefore, it is important to identify anticancer substances that are present in foods and can be ingested in adequate amounts. Although CA, a polyphenolic compound found in foods, is not an established antitumor chemotherapeutic agent, several studies have

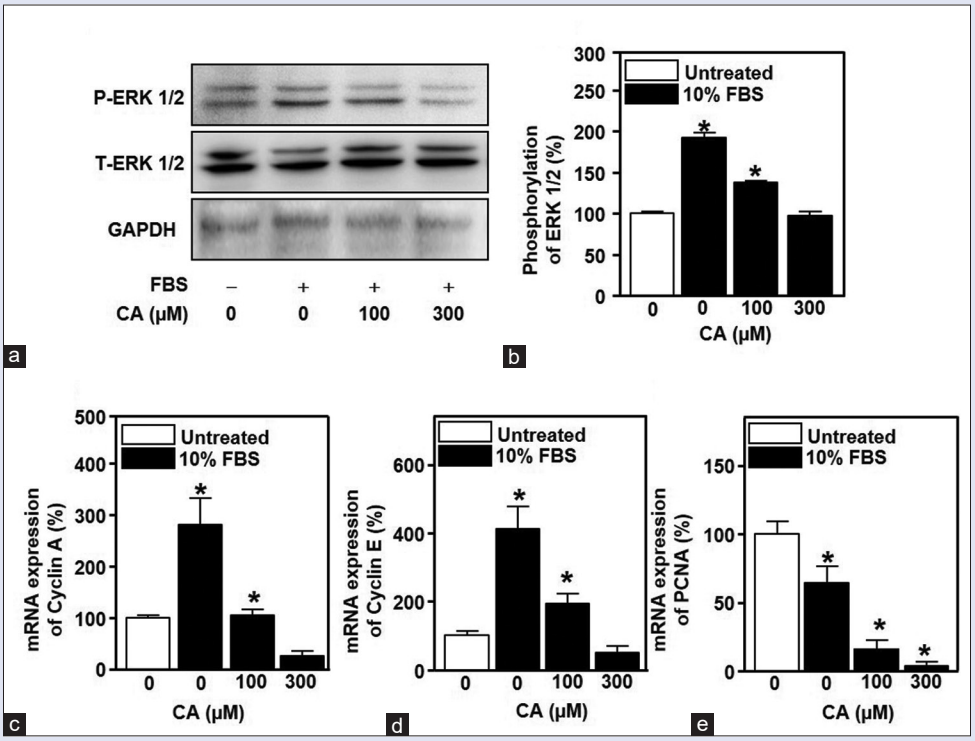

Figure 4: Effect of chlorogenic acid on extracellular signal-regulated kinase ½ phosphorylation and cell cycle regulators in C6 glioma cells. C6 glioma cells were seeded in 100 mm dishes and incubated with serum-free medium for 24 h. The cells were treated with chlorogenic acid (100 and 300 μM) for 24 h. (a) Cell lysates were separated on 12% acrylamide gels and the level of ERK½ phosphorylation was measured by chemiluminescence. (b) The graphs are based on the band intensities from panel A. The intensity obtained from untreated cells was considered to be 100%. The data are presented as mean ± standard deviation ($n = 3$). (c-e) After total RNA extraction, real-time polymerase chain reaction analysis was performed. The values of relative gene expression were determined by calculating the value of the Δcycle threshold (ΔCt), normalizing the average Ct value to the control gene, glyceraldehyde 3-phosphate dehydrogenase, and then calculating the $2^{-\Delta\Delta Ct}$ values. For each gene, the level of expression in untreated cells was considered as 100%. The data are presented as mean ± standard deviation ($n = 3$). *$P < 0.05$ compared with untreated cells. CA: Chlorogenic acid; FBS: Fetal bovine serum; ERK; extracellular signal-regulated kinase

demonstrated its anticancer effects in HepG2, Panc-1, MBA-MB-231, and HT-29 cells.[26-29] Moreover, CA is known to exert beneficial effects on human heath, including anticancer, anti-inflammatory, and antioxidant effects.[30,31] However, whether CA exerts antitumor effects on glioma is unknown. Therefore, in this study, we tested the anticancer effects of CA on glioma cells, and based on our results, we suggest that CA is a candidate medicinal food chemical that can regulate glioma.

Currently, one of the most interesting approaches to optimize methods in the accumulation of active compounds from the plant is application of culture *in vitro*. Kowalczyk *et al*. reported the alteration of anticancer compounds from *Menyanthes trifoliata* L. *in vitro* culture.[32] In addition, these results are also consistent with the possibility of increasing anticancer substances *in vitro* culture of the plants in our study.

In this study, owing to the paucity of *in vitro* studies, it is difficult to estimate the effective anticancer dose of CA. To address this issue, we tested the effects of different CA concentrations on the expression of cell cycle regulators and the phosphorylation of ERK ½ *in vitro*. In addition, we investigated the anticancer effect of CA on the migration and growth of C6 glioma cells. Interestingly, CA significantly reduced the expression of cell cycle-related factors, as well as the phosphorylation of ERK½. Moreover, CA exhibited an inhibitory effect on the migration and proliferation of C6 glioma cells. These findings suggest that CA could be a regulator of malignant glioma.

Elevated levels of cell cycle-related factors and phosphorylated ERK½ are biomarkers of cancer cell activation.[33] Our results imply that CA treatment attenuated cell division signals involving cyclin E, cyclin A, and PCNA PCNA [Figure 4c, d and e]. Increased production of ROS and the consequent oxidative stress contribute to, or accompany, the progression of several diseases. ROS may alter important cellular activities by inducing or inhibiting specific transcription factors and modifying receptor signaling.[34] In addition, high levels of ROS can cause extensive cell death and apoptosis.[35] In the current study, we showed that CA treatment significantly upregulated ROS levels in glioma cells. Overall, our results suggest that CA can be effectively used as an anticancer agent. Furthermore, Lee K *et al*. suggested that CA may be useful in controlling brain disorders, as it can cross the blood–brain barrier,[36] thus supporting our observation.

CONCLUSION

We found that CA could significantly modulate cell cycle and migration, likely by controlling the expression of factors such as cyclin E, cyclin A, and PCNA and phosphorylation of ERK½. In light of these findings, we suggest that CA may be used as an anticancer drug to prevent abnormal migration and proliferation of glioma cells.

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Conflicts of interest

There are no conflicts of interest.

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