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Cytochalasin E, a Potential Agent for Anti-Glioma Therapy, Efficiently Induces U87 Human Glioblastoma Cell Death

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***SUMMARY*. Glioblastoma is one of the most malignant brain tumors. Current treatments for glioblastoma usually make poor responses, and novel treatment strategies are extremely imperative. Cytochalasin E was reported to inhibit angiogenesis and tumor growth in some studies, but its effects on gliomas are still unknown. In this study, we found cytochalasin E inhibits U87 human glioblastoma cell growth in a very low concentration range of 10^{-8} to 10^{-6} M in a time and concentration dependent manner, and the IC50 were $1.17 \pm 0.32 \times 10^{-7}$ M for 48 h treatment, $6.65 \pm 1.12 \times 10^{-8}$ M for 72 h and $3.78 \pm 1.30 \times 10^{-8}$ M for 96 h. We also found cytochalasin E induces cell-cycle G2/M phase arrest (72 h-treatment of 10^{-6} M cytochalasin E caused 56.2 ± 6.1 % cells arrest in G2/M phase) and cell apoptosis (96 h-treatment of 10^{-6} M cytochalasin E induced 24.1 ± 4.2 % cells apoptosis). Thus, cytochalasin E is proposed as a potential agent for glioblastoma chemotherapy.**

INTRODUCTION

Gliomas are the most common tumors in central nerve system, most of them malignant [1]. Local control with surgery, radiation, and chemotherapy remain the pillars of treatment for high-grade gliomas, such as glioblastoma [2]. It is abetted to discover novel agents with high cytotoxicity and low side effects for glioblastoma therapy.

Cytochalasins are a family of compounds with diverse activities on cellular function, including inhibition of actin polymerization and glucose transport [3]. The effect of cytochalasins on actin polymerization has been widely studied. Cytochalasin E (Fig. 1) is a unique epoxy-containing and more potential cytochalasin. It has been reported to be a novel inhibitor of angiogenesis and tumor growth [4]. However, no reported studies ever mentioned the effects of cytochalasin E on human gliomas. We hypothesized that cytochalasin E may be a potential agent for glioblastoma therapy. Since the anti-actin effect of cytochalasin E is another way to disrupting cytoskeleton which is similar to anti-tubulin effect of paclitaxel or vincristine, in this study, we investigated the effects of cytochalasin E on cell cycle progression and cell apoptosis in U87 human glioblastoma cells.

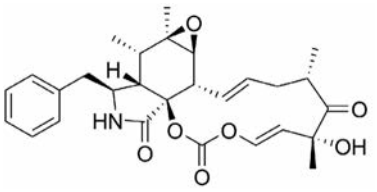

Figure 1. Structure of cytochalasin E.

MATERIALS AND METHODS

Agent treatment

Cytochalasins E was purchased from AppliChem (Darmstadt, Hesse, Germany). In all assays, agent was dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted in serum-supplemented medium immediately before use. DMSO concentrations never exceed 0.1 % (v/v).

KEY WORDS: Actin, apoptosis, Cytochalasin E, Glioma, Proliferation.

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Cell line and culture

U87 human glioblastoma cell line was obtained from American Type Culture Collection (Manassas, VA, USA), and maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % fetal bovine serum, glutamine, nonessential amino acids, and 1 % penicillin/streptomycin (complete medium). Cells were grown at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2.

WST-8 cell viability assay

WST-8 cell viability assay was performed using the cell counting kit-8 (CCK-8) from Dojindo Laboratories (Kumamoto, Kyushu, Japan) and according to the manufacturer's instructions. U87 cells were plated in a 96-well plate at a density of 3000 cells/well in 200 µL of culture medium together with cytochalasin E dilution. Then, the cells were cultured in humidified incubator containing 5 % CO_2. After 48, 72 or 96 h, CCK-8 solution was added to each well and incubated for one hour in the incubator. The absorbance measurement was executed at 450 nm using an enzyme-linked immunosorbent assay plate reader (Bio-Rad Laboratories, Inc., Berkeley, CA, USA).

Phalloidin staining

U87 cells (20,000 cells/well) were plated and incubated overnight on circular coverslips in 24-well culture dishes, and then be treated with cytochalasin E for 24 h. After that, cells were washed with PBS and then fixed with 4 % paraformaldehyde in PBS for 30 min at room temperature. The fixed cells were washed twice with PBS and then permeabilized for 20 min at room temperature in PBS containing 0.5 % Triton X-100. The permeabilized cells were incubated with 1 mM tetramethylrhodamine isothiocyanate (TRITC)-phalloidin (Beyotime, Shanghai, China) for 60 min at 37 °C. The labeled cells were washed twice with PBS and then mounted onto slides. Pictures were captured by using fluorescence microscope.

Cell-cycle analysis by flow cytometry

For flow cytometric analysis, cells were plated in 10 cm culture dishes and subsequently exposed to cytochalasin E for 48 or 72 h at 37 °C. Cells were subsequently collected by trypsinization, centrifuged (3500 rpm for 5 min), and washed twice with PBS. Cells were fixed by 1 mL ethanol (70 %) and pelleted by centrifugation (3500 rpm for 5 min), rinsed twice with PBS, and incubated with propidium iodide. A total of 10,000 nuclei were analyzed in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Apoptosis assay

Apoptosis was evaluated in U87 cells with flow cytometry using annexin V-FITC conjugates and propidium iodide staining. Briefly, cells were collected and centrifuged, the supernatant was discarded, and the pellet was incubated for 15 min at room temperature with annexin V-FITC and propidium iodide before analysis with a FACSAria III flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical analysis

All data were analyzed by oneway analysis of variance (ANOVA) and the Student-Newman-Keul's test, and expressed as mean ± standard deviation (SD). Results at $P < 0.05$ were considered significant.

RESULTS

Cytochalasin E inhibited U87 cell growth

U87 cells were respectively treated with 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} or 10^{-6} M cytochalasin E for 48, 72 or 96 h. The O.D. value was detected through CCK-8 assay to represent the quantity of viable cells. Cytochalasin E inhibited U87 cell growth in 10^{-8} to 10^{-6} M concentration range; whereas inhibition was not observed at concentrations lower than 10^{-9} M. As shown in Figure 2, the inhibitory effect of cytochalasin E on cell growth was time and concentration de-

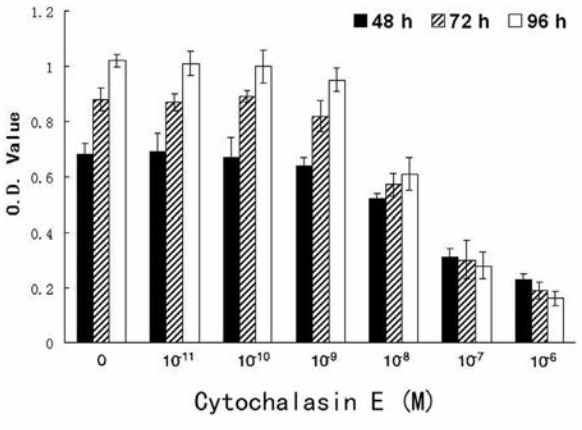

Figure 2. Bar graphs representing effect of cytochalasin E on U87 cell growth. Cytochalasin E was used at concentration from 10^{-11} M to 10^{-6} M with 48, 72, or 96 h of incubation. Quantity of the viable cells was evaluated by measuring absorbance (optical density, O.D.) at 450 nm. All data are expressed as mean and S.E. from three independent experiments

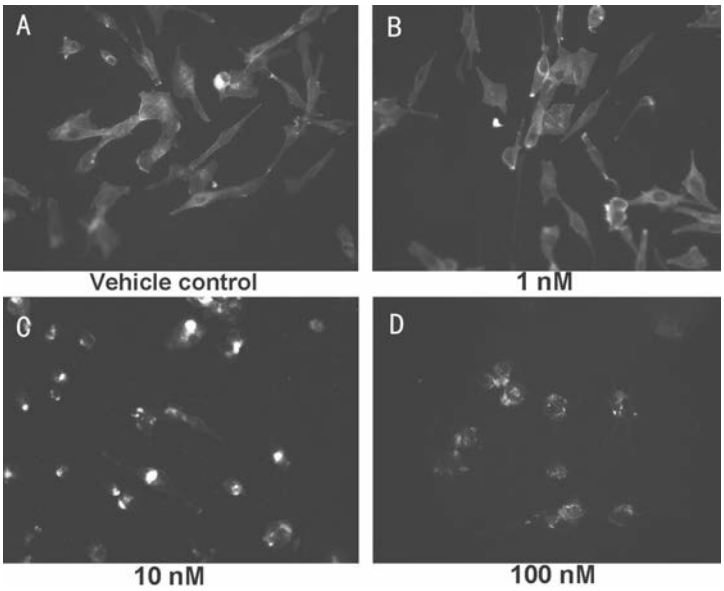

Figure 3. The effect of cytochalasin E on actin polymerization of U87 cells. Cells were fixed and stained with TRITC-phalloidin. Immunofluorescence photographs were taken at 200× magnification. **A**) Vehicle control; **B**) U87 cells treated with 10^{-9} M cytochalasin E, no disruption of actin cytoskeleton was observed; **C**) Cells treated with 10^{-8} M cytochalasin E. The anti-actin effect of cytochalasin E was revealed by the changes of cell morphology and disorders of actin fibers; **D**) Cells treated with 10^{-7} M cytochalasin E. The anti-actin effect became pronounced with the absence of stress fiber staining and retraction of the cytoplasm.

pendent. The IC50 were 1.17 ± 0.32 × 10^{-7} M (48 h), 6.65 ± 1.12 × 10^{-8} M (72 h) and 3.78 ± 1.30 × 10^{-8} M (96 h).

Cytochalasin E disrupted actin cytoskeleton of U87 cells

U87 cells treated with cytochalasin E were also stained with phalloiden to visualize the effect of cytochalasin E on actin. Disruption of actin stress fibers was not observed until 10^{-8} M cytochalasin E were used (Fig. 3). The anti-actin effect of cytochalasin E was revealed by the changes of cell morphology and disorders of actin fibers at concentration of 10^{-8} M (Fig. 3C), and became pronounced at 10^{-7} M with the absence of stress fiber staining and retraction of the cytoplasm (Fig. 3D).

Effects of cytochalasin E on cell cycle progression and apoptosis

To further investigate the effects of cytochalasin E on U87 cell, cell cycle and apoptosis analysis were performed. Cells were treated with increasing concentrations of cytochalasin E for the same times as described in Materials and Methods section. Cell cycle progression was evaluated after propidium iodide staining by fluorescence-activated cell-sorting analysis. For U87 cells, after treatment with 10^{-9} M cytochalasin E for 72 h or 96 h, no alteration of cell cycle progression was observed comparing with control condition (Fig. 4A-C). But, after treatment with 10^{-6} M to 10^{-8} M cytochalasin E for 72 h, the proportion of cells in the G2/M phase increased in a concentration-dependent manner from 27.5 ± 4.8 % (10^{-8} M) to 38.3 ± 3.2 % (10^{-7} M) and to 56.2 ± 6.1 % (10^{-6} M), with a corresponding decrease in cells in the G0/G1 and S phases (Figure 4D-F). After 96h-treatment with 10^{-6} M cytochalasin E, the proportion of cells in Sub-G1 phase (18.9 ± 2.2 %) which represented apoptosis significantly increased comparing with the 72h-treatment condition (12.1 ± 1.8 %). This indicated a time-dependent apoptosis induced by 10^{-6} M cytochalasin E (Figs. 4F and G).

Cytochalasin E induced apoptosis in U87 cells was also detected by means of annexin V-FITC conjugates and propidium iodide staining. As shown in Figure 4, in vehicle control cells the apoptosis proportion was only 4.3 ± 0.8 % (Fig. 4H), and in cells treated with 10^{-6} M cytochalasin E for 96 h the apoptosis proportion increased into 24.1 ± 4.2 % (Fig. 4I).

DISCUSSION

Glioblastoma, one of the most malignant gliomas, is very difficult to treat because of its aggressive and wide invasion into the surrounding normal brain tissue. Although surgical and radiotherapeutic techniques had been improving, and temozolomide had been applying in the multimodal treatment strategy, the prognosis of patients with glioblastoma remains poor [5,6]. The median overall survival rate is approximately 15 months, 88 % of patients died within 3 years [7]. Thus, it is worth to develop novel agents with high cytotoxicity and low side effects for glioblastoma therapy.

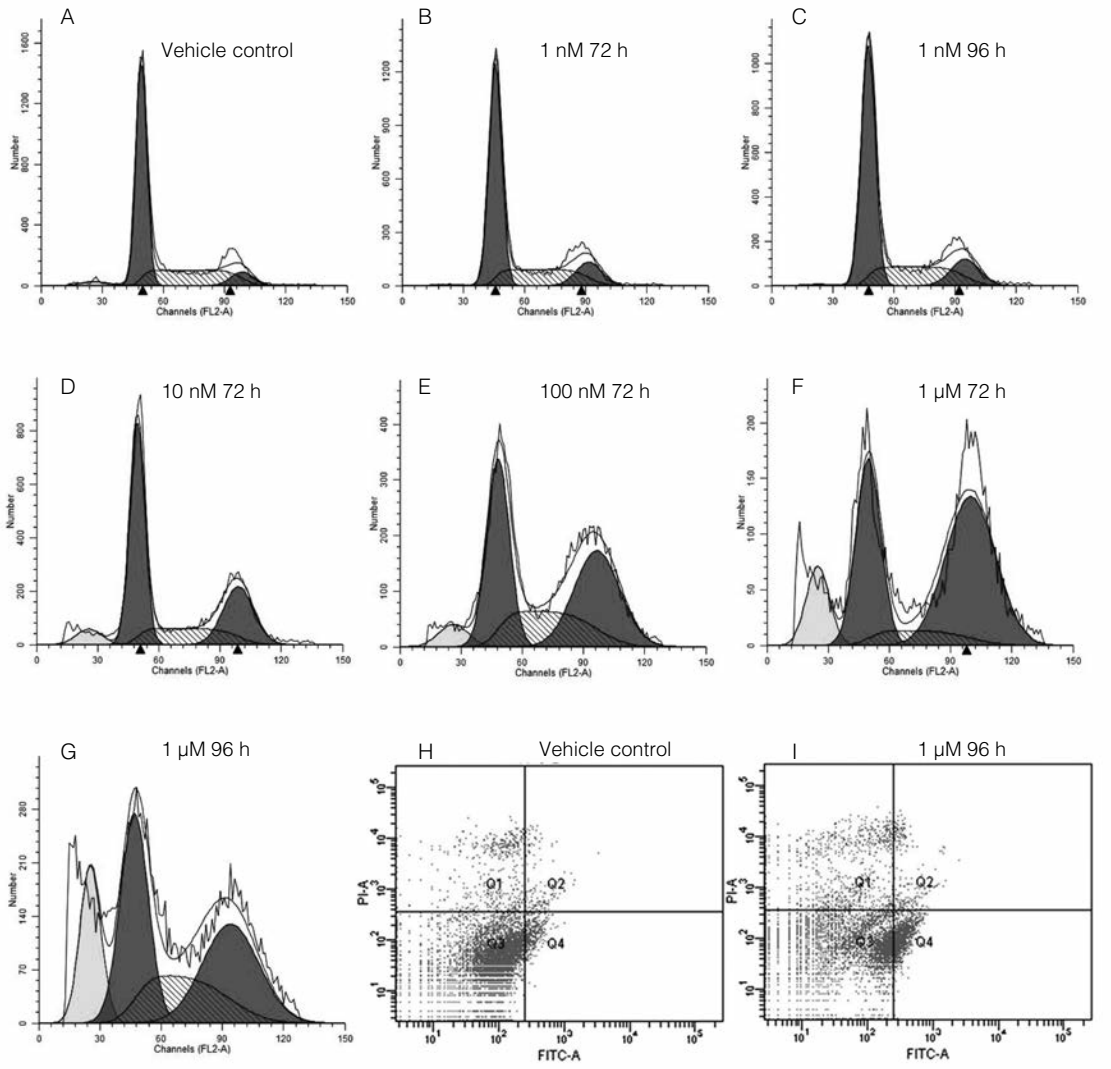

Figure 4. Cell-cycle analysis and apoptosis assay of U87 cells: (**A**) Vehicle control. (**B, C**) Cells treated with 10^{-9} M cytochalasin E for 72 h and 96 h. (**D-F**) Cells treated with 10^{-8} M, 10^{-7} M, or 10^{-6} M cytochalasin E for 72 h. (**G**) Cells treated with 10^{-6} M cytochalasin E for 96 h. (**H-I**) Apoptosis assay by means of annexin V-FITC conjugates and propidium iodide staining for cells non-treated or treated with 10^{-6} M cytochalasin E for 96 h.

Cytochalasin E is an epoxide-containing cytochalasin family member which was isolated as a minor metabolite of the food storage mold *Aspergillus clavatus* [8]. A previous study shown that cytochalasin E exhibited a unique biphasic inhibition of bovine capillaries epithelial cell proliferation that was not observed using other nonepoxide cytochalasin analogs *in vitro,* and an inhibition of angiogenesis and tumor growth *in vivo* [4]. However, no reported studies ever mentioned the effects of cytochalasin E on human gliomas. In our study, we found that 10^{-8} M or more cytochalasin E could efficiently inhibit U87 cell growth, induce cell-cycle G2/M phase arrest and apoptosis. The cell cycle consists of four distinct phases: G1 phase, S phase (synthesis), G2 phase (collectively known as interphase) and M phase (mitosis). M phase composed of two coupled processes, mitosis and cytokinesis. In process of cytokinesis, actin cytoskeleton is essential to promote cytoplasm dividing in half and forming distinct cells. Disruption of actin cytoskeleton induced by cytochalasin E stopped the process of cytokinesis and caused the cell-cycle G2/M phase arrest. It was considered that cell cycle arrest at the G2/M phase often precedes the onset of apoptosis [9,10]. In this study it was detected a time-dependent apoptosis increase induced by 10^{-6} M cytochalasin E accompanying with a corresponding decrease in G2/M

arrest (Figs 4F and G). This indicated that cytochalasin E induces cell cycle arrest earlier and subsequently causes cell apoptosis.

At the cytotoxic concentrations, cytochalasin E also induced disruption of actin cytoskeleton with the absence of stress fiber staining and retraction of the cytoplasm (Fig. 3). Actin exists in all eukaryotic cells as components of the cytoskeleton and participates in many important cellular processes including cell motility, cell division and cytokinesis, cell signaling, the establishment and maintenance of cell junctions, and so on [11,12]. Similar to tubulin, anther component of the cytoskeleton, actin can also be a valid target for anti-tumor therapy. Anti-tubulin agents such as paclitaxel and vincristine are being wildly used for chemotherapy [13,14], but no anti-actin agent has been developed or used to treat tumors. In the present study, as a highly efficient actin inhibitor, cytochalasin E was indicated to induce glioblastoma cell death in a very low concentration (low than 1 μM), and exhibit a great potential for glioma therapy. Further studies involved in the *in vivo* potency and pharmacokinetic properties of cytochalasin E are necessary, and more investigations are also needed for detecting specific molecular targets of cytochalasin E and revealing related signaling pathways.

CONCLUSIONS

In summary, the obtained results provide the first experimental evidence that cytochalasin E inhibits human glioblastoma cell growth through cell cycle arrest and apoptosis-induction. Cytochalasin E is proposed to be a potential agent for future glioblastoma therapy.

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