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Cordycepin inhibits migration of human glioblastoma cells by affecting lysosomal degradation and protein phosphatase activation

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Abstract

Cordycepin, a nucleoside-derivative-isolated form *Cordyceps militaris*, has been reported to suppress tumor cell proliferation and cause apoptosis. This study investigates the effect of cordycepin on the migration of human glioblastoma cells. Cordycepin suppressed the migration of the human glioblastoma cell lines U87MG and LN229 in transwell and wound healing assays. Cordycepin decreased protein expression of integrin α1, focal adhesion kinase (FAK), p-FAK, paxillin and p-paxillin. The lysosomal inhibitor NH_4Cl blocked the ability of cordycepin to inhibit focal adhesion protein expression and glioma cell migration. In addition, the protein phosphatase inhibitors calyculin A and okadaic acid blocked the cordycepin-mediated reduction in p-Akt, p-FAK and migration. Hematoxylin and eosin staining of mouse xenografts demonstrated that cordycepin reduced brain tumor size *in vivo*. In conclusion, cordycepin inhibited migration of human glioblastoma cells by affecting lysosomal degradation and protein phosphatase activation. This pathway may be a useful target for clinical therapy in the future.

1. Introduction

Gliomas are primary malignant brain tumors that develop from the supporting glial cells of the central nervous system. Gliomas invade the surrounding brain tissue, making them difficult to treat. The therapeutic management of glioblastoma includes maximal surgical resection and concurrent chemoradiotherapy. However, the mean survival of glioblastoma patients is only approximately 15 months [1]. Therefore, it is important to identify better ways to treat gliomas.

Cordycepin, isolated from cultured *Cordyceps militaris*, inhibits cancer growth [2], inflammation [3] and platelet aggregation [4]. The molecular mechanisms by which cordycepin inhibits cancer have been reported. Cordycepin suppressed migration and integrin/focal adhesion kinase (FAK) signaling in hepatocellular carcinoma [5,6]. Cordycepin also reduced migration and invasion by regulating tight junction activity and tissue inhibitors of metalloproteinases (TIMPs) in human prostate carcinoma cells [7]. Moreover, cordycepin blocked TNF-α-induced migration and invasion through inhibition of matrix metalloproteinases-9 (MMP-9), nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) in human bladder cancer cells [8]. In a previous study, we demonstrated that cordycepin induces apoptosis in C6 glioma cells via the adenosine 2A receptor-p53-caspase-7-PARP cascade [2]; however, the effect of cordycepin on migration of glioblastoma cells remains unclear.

Cellular migration and invasion are important and complex processes that lead to glioblastoma progression. Integrin β1 and focal adhesion kinase (FAK) are involved in the formation of focal adhesion complexes, which control cell adhesion and migration [9]. Integrins are heterodimeric membrane proteins containing α and β subunits. Clustering of integrins leads to FAK activation and focal adhesion formation, which subsequently activates cellular signaling pathways [10]. Moreover, the expression of FAK is up-regulated in human anaplastic astrocytoma and glioblastoma [11]. Inhibition of integrin and FAK both block the migration of glioblastoma cells [12,13]. Targeting of focal adhesion complexes may be a potential treatment for glioblastoma. Therefore, this study aims to clarify the effect of cordycepin on cell migration in human glioblastoma both *in vitro* and *in vivo*.

2. Materials and Methods

2.1. Cell culture

Human glioma cell lines U87MG and LN229 were purchased from the American Type Culture Collection (Rockville, MD, USA). U87MG-Luc2 cells were derived via the stable transfection of pLuc2-iRFP and selected with a FACSAria Fusion Sorter. The cells were cultured with Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% fetal bovine serum (both from Gibco BRL, Grand Island, NY, USA), 1 mM sodium pyruvate (Sigma, St. Louis, MO, USA) and 100 IU/ml of penicillin and streptomycin (pH 7.4) (Gibco BRL, Grand Island, NY, USA) in a humidified atmosphere of 5% CO_2–95% air in a 37°C incubator.

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2.2. Drugs

Cordycepin, 2,3,3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and protein phosphatase inhibitors calyculin A and okadaic acid were purchased from Sigma (St. Louis, MO, USA). NH_4Cl and MG132 were purchased from Calbiochem (VWR International, Radnor, PA, USA).

2.3. Cell survival assay

U87MG and LN229 glioma cells were plated at 2×10 [4] cells per well in a 24-well plate and incubated for 24 h to allow cells to adhere. Then, the cells were cultured for 24 h in the presence of varying concentrations of cordycepin or 0.1% dimethyl sulfoxide (DMSO, Sigma) as a control. After two washes with phosphate-buffered saline (PBS) (137-mM NaCl, 2.7-mM KCl, 1.5-mM KH_2PO_4, 8-mM Na_2HPO_4, pH 7.4), DMEM medium containing 0.5 mg/ml of MTT was added to each well, and incubation continued for another 4 h. The reaction solution was then carefully removed, and the cells were lysed with DMSO. The absorbance at 590 nm was measured with a spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA).

2.4. Migration assay

Cell migration was assessed using the transwell migration and wound healing assays. The transwell migration assay was conducted by seeding 5×10^4 U87MG or LN229 glioma cells in the upper chamber of a transwell (Costar, Acton, MA, USA). After cell attachment, cells were grown in medium either with or without cordycepin for 24 h. The cells on the lower side were fixed in 10% formalin and stained with Coomassie brilliant blue G-250 (Sigma, St. Louis, MO, USA). The number of migrated cells was counted in three randomly selected fields from each membrane, with the membranes examined at three time points in each experimental group. The wound healing assay was performed by growing cells to confluence to form a monolayer. The monolayer was then scratched using a P200 pipette tip. After 24 h of treatment with cordycepin, the monolayer was photographed to visualize wound healing. Images shown are representative of three different experiments.

2.5. Western blotting

After various treatments, the U87MG or LN229 glioblastoma cells were washed once with PBS and then homogenized in lysis buffer (10 mM EGTA, 2 mM MgCl2, 60 mM PIPES, 25 mM HEPES, 0.15% Triton X-100, 1 μg/ml of pepstatin A, 1 μg/ml of leupeptin, 1 mM NaF and 1 mM phenylmethylsulfonyl fluoride). Protein samples (60 μg per lane) were electrophoresed on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad Life Science). Strips from the membrane were blocked with 5% nonfat milk in Tris-buffered saline, pH 8.2, containing 0.1% Tween (TBS-Tween) and incubated overnight at 4°C with a 1:500 dilution of rabbit antibodies against phosphorylated-FAK (p-FAK), phosphorylated-paxillin (p-paxillin), Akt, phosphorylated-Akt (p-Akt) (Cell Signaling), GAPDH (BioVision Research Products, Mountain View, CA, USA) or mouse

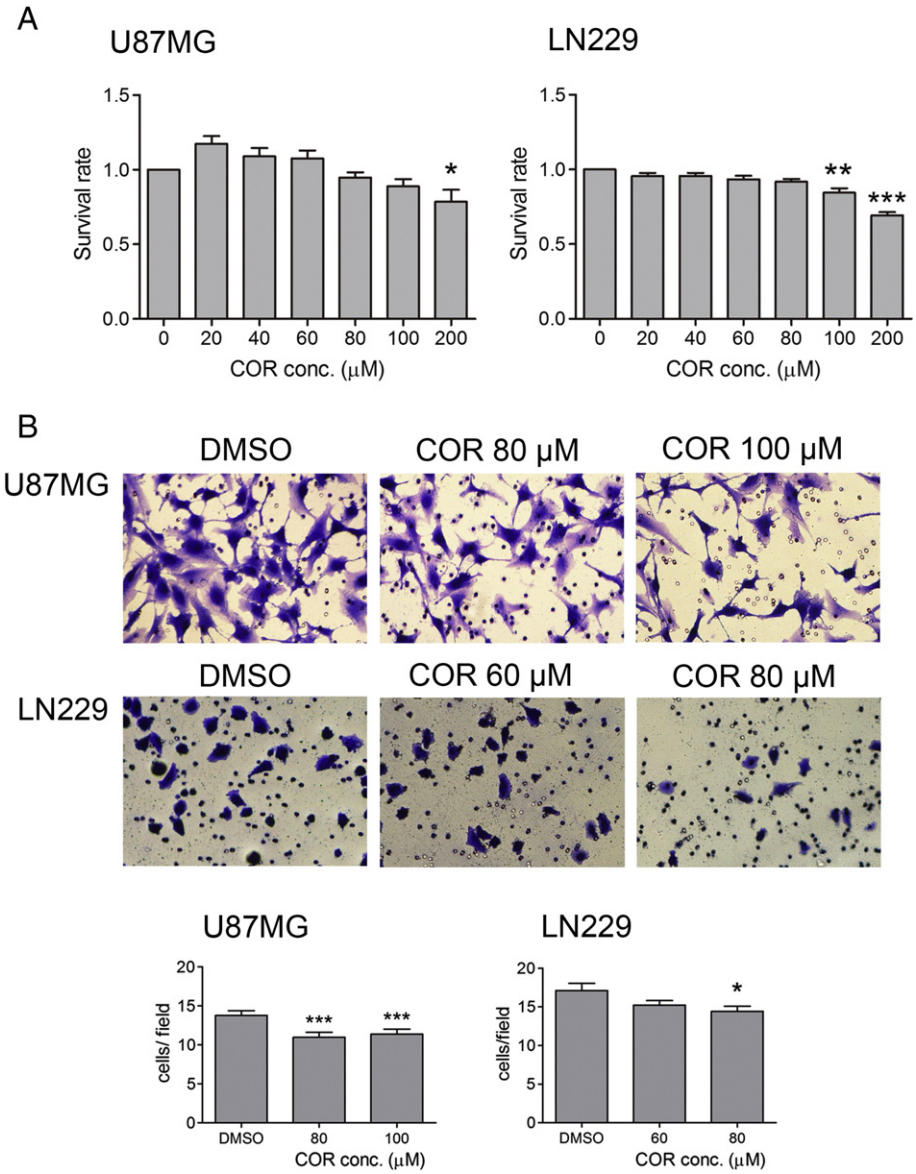

Fig. 1. The effects of cordycepin on viability and migration of human U87MG and LN229 glioblastoma cells. (A) Cells were treated with DMSO or different concentrations of cordycepin for 24 h, and the survival rate was analyzed by the MTT test. Values are expressed relative to the control group. *, $P<.05$; **, $P<.01$ or ***, $P<.001$. (B) Cells were cultured in the upper chamber of a transwell for 1 h to allow adhesion. After 24 h of incubation with or without cordycepin, the migrated cells in the lower chamber were counted. *, $P<.05$; **, $P<.01$ or ***, $P<.001$ compared to the control group.