Protein Science Key Laboratory of the Ministry of Education[1], Department of Biological Sciences and Biotechnology, School of Medicine, Tsinghua University, Beijing Institute of Organic Chemistry[2], Jiangxi Science & Technology Normal University, Nanchang, China

Echinacoside retards cellular senescence of human fibroblastic cells MRC-5

HONG XIE[1], HUI ZHU[1,2], CONG CHENG[1], YU LIANG[1], ZHAO WANG[1]

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Prof. Dr. Zhao Wang, Protein Science Key Laboratory of the Ministry of Education, Department of Biological Sciences and Biotechnology, School of Medicine, Tsinghua University, 100084, Beijing, China
zwang@tsinghua.edu.cn

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In this study, effects of echinacoside, one of the phenylethanoids isolated from the stems of *Cistanches salsa*, a Chinese traditional herbal medicine, on human embryo lung fibroblastic MRC-5 cells, was investigated. Activity of cell proliferation was evaluated with Alamar Blue, showing that treatment with echinacoside could retard the senescence. Flow cytometry results show that echinacoside could trigger cells in the G1 phase to enter the S phase and G2 phase, and could improve ROS degradation. The results from comet assay indicate that echinacoside could protect cells from DNA damage, partly elucidating the mechanism of its effects. All of the above results suggest that echinacoside has potential anti-senescence activity.

1. Introduction

Normal cells cultured *in vitro* proliferate for a limited number of population doublings (the 'Hayflick limit') and enter a stage of replicative senescence. Senescent cells undergo cell growth arrest in the G1 phase and a change in morphology and metabolism, including cellular enlargement, increased lysosome biogenesis, and expression of higher β -galactosidase activity at pH 6 (senescence-associated β -galactosidase, SA- β -Gal) (Hayflick and Moorhead 1961; Dimri 1995). The accumulation of senescent cells prognosticates the age-related decline in tissue/organ functions. To access the way of delaying or reversing aging, efforts to discover active anti-aging substances have never ceased.

Echinacoside is one of the phenylethanoids isolated from stems of *Cistanches salsa*, a Chinese traditional herbal medicine, which has potential biological activities, both as an antisenium and antifatigue agent (Deng et al. 2004; Xiong et al. 1996). It has also been reported to behave *in vitro* as a potent free radical scavenger (Facino et al. 1995), and exhibits neuroprotective activities *in vitro* and *in vivo* (Koo et al. 2005; Geng et al. 2007). Echinacoside may be a candidate for an anti-aging substance, and an anti-aging activity might be one of its biological properties.

Thus, we treated a senescent human embryo lung fibroblastic cell line MRC-5 with echinacoside, expecting it could retard or reverse the senescence.

2. Investigations and results

Firstly, we added echinacoside into culture medium to check its influence on cell growth and cell cycles. It was observed that the viability of the cells with 48 h exposure to echinacoside increased evidently relative to the control, as the concentration increased (Fig. 1A), indicating that echinacoside helped senescent cells proliferate. Result of SA- β -Gal staining showed that the level of SA- β -Gal decreased obviously after treatment of echinacoside (data not shown), suggesting that a part of senescent cells were reversed from senescence. To confirm whether senescent cells were indeed propelled to proliferate by echinacoside, flow cytometry was introduced to check the cell cycle phase alteration of the senescent MRC-5 cells after treatment with echinacoside. In our study, we observed that with the concentration of Echinacoside increased, the ratio of cells in the G1 period was reduced gradually. On the contrary, more cells were driven to enter the S phase and G2 phase (Fig. 1B). The accumulation of senescence-associated heterochromatin foci (SAHF) is another specific biomarker of senescent cells (Shay and Wright 2001). Senescent cells displayed punctuated DNA foci which were visualized by Hoechst staining (Fig. 1C). In contrast, MRC-5 cells treated with echinacoside appeared to have less SAHF.

The level of reactive oxygen species (ROS) increases as cells approach the Hayflick limit (Hutter et al. 2002). Continuous treatment of cells with sub-lethal levels of ROS not only increases the level of oxidative damage products such as lipofuscin (Sitte et al. 2001), but also causes permanent growth arrest accompanied by the activation of the p53-growth inhibitory pathway (Toussaint et al. 2000). Therefore, at the cellular level, ROS play a key role in inducing senescence. The fluorescent dye DCFH-DA was used to measure ROS contents with flow cytometry. It was shown that compared with the control, the ROS contents were decreased evidently in cells incubated with echinacoside (Fig. 2A). These results indicate that echinacoside might act as an anti-oxidant, lowering ROS production or accelerating ROS removal.

Accumulated reactive oxygen species (ROS) cause the remarkable impairment of macromolecules in cells, including proteins, DNA and telomeres (Kovtun et al. 2007). Oxidative DNA damages trigger p53-dependent G1 growth arrest, following the expression of $p21^{Waf\text{-}1/SDI\text{-}1/Cip1}$ (Chen et al. 1998) and cell

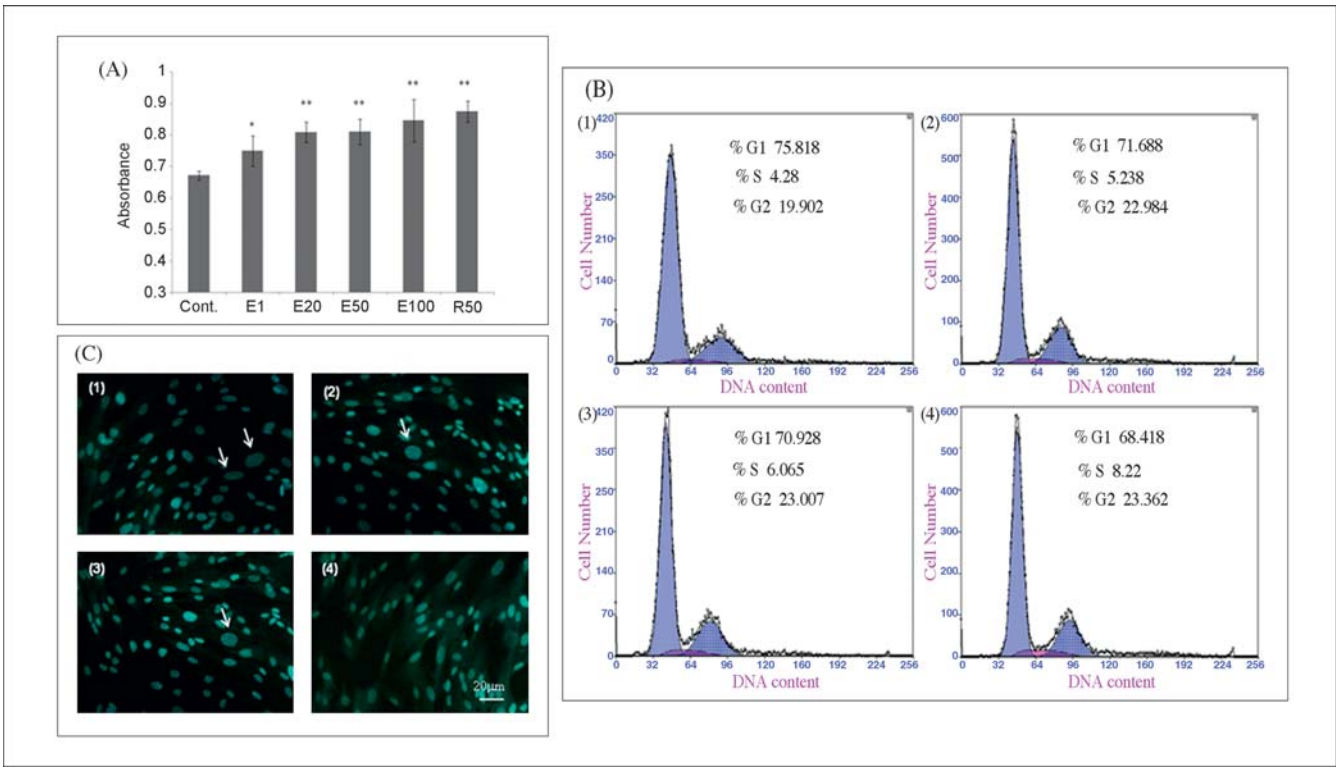

Fig. 1: Echinacoside retards cellular senescence of MRC-5 cells.
A. Effects of echinacoside on cell proliferation. Histograms in the figure represent non-treated cells, cells treated with 1, 20, 50, 100 μM of echinacoside respectively, and cells treated with 50 μM resveratrol as positive control. * $p < 0.05$; ** $p < 0.01$. Three independent assays were performed, and data shown are the mean ± S.D. Analyzed by onetailed t test. B. Effects of echinacoside on cell cycle. (1) Non-treated MRC-5 cells at late cell population doublings; (2), (3) and (4) represent cells treated with echinacoside at 1, 50 and 100 μM, respectively. Two independent assays were performed and one typical result was presented. C. Senescence-associated heterochromatic foci (SAHF), another classical marker of senescence, was visualized by Hoechst staining in MRC-5. (1) Non-treated MRC-5 cells; (2), (3) and (4) represent cells treated with echinacoside at 1, 50 and 100 μM, respectively. Scale bars were equal to 20 μm

senescence in normal human fibroblasts. We used comet assay to measure the DNA damage and its repair. The size of DNA fragments and the number of breaks determine the migration and pattern of the comet seen. Tail DNA content (product of tail length and tail DNA content) increases with damage. Our results show that pretreatment with echinacoside can partly promote the repair of DNA damage, at least protect the cells from DNA lesions caused by H_2O_2 (Fig. 2B).

Based on these facts, we conclude that echinacoside has anti-senescence activity at least in MRC-5 cells. The results of our study show that echinacoside can reduce ROS accumulation, protect cells from DNA damage, promote MRC-5 cells from the G1 phase into the S and G2 phases, and thus improve proliferation of the cells. Generally speaking, the effects of echinacoside on MRC-5 cells could be divided into two aspects: to promote cell proliferation and to reduce ROS accumulation. Although the precise mechanism of cell senescence is still unknown and the network of cell cycle regulation remains controversial, we have demonstrated that echinacoside can protect cells from oxidative damage. We therefore speculate that echinacoside might be a good candidate for regulating senescence, even though the precise underlying mechanism remains an open question and further studies are in progress.

3. Experimental

3.1. Cell culture and treatment

Human embryo lung fibroblastic cell line MRC-5 (Institute of Biochemistry and Cell Biology, SIBS, CAS) was maintained in modified minimum essential medium (Gibco/BRL) and supplemented with 10% fetal bovine serum. MRC-5 cells undergo replicative senescence after multiple cell passages, assessed by the slowing of metabolism and cessation of division within 7 days. All cultures were seeded at a cell density of $10^4/cm^2$ unless otherwise noted, and were allowed to proliferate, undisturbed, for 7 days. Various concentrations of echinacoside were added 48 h before examination with solvent as control, unless otherwise stated. The concentration of DMSO in cultures was less than 0.3% (v/v). For H_2O_2 treatment, cells were induced with 250 μM H_2O_2 for 12 h.

3.2. Survival rate assay

Cells were cultured and treated with echinacoside on 96 well plate. Before detection, 10% Alamar Blue™ was added to the medium and incubated for 4 h. Absorbance was measured with spectrophotometer.

3.3. Flow cytometry analysis

Flow cytometry was introduced to measure the DNA contents and intracellular ROS levels using propidium iodide and DCFH-DA respectively. Cells were plated at a density of 10^5 cells/10 ml cell solution in 100 mm diameter dishes and supplemented with echinacoside for 48 h after cell seeding. After 48 h treatment, cells were harvested and washed in PBS after centrifugation, then resuspended in PBS solution with 0.1% RNase and 50 μg/ml propidium iodide or culture medium containing 10 μM DCFH-DA for 30 min. DNA or ROS contents were then determined by fluorescence-activated cell sorting on a Beckman Coulter Flow Cytometry System. The data were analyzed by MultiCycle software.

3.4. Hoechst staining

Cells were cultured and treated with echinacoside for 48 h on a 96 well plate. Medium was removed, Cells were washed with PBS, then fixed with 4% formaldehyde in PBS for 20 min, followed with washing with PBS once, and stained with Hoechst dye for 10 min. Pictures were captured.

3.5. Single-cell gel electrophoresis (comet assay)

Comet assay was conducted as reported (Olive and Banath 2006). Individual comet images were analyzed with CometScore software.

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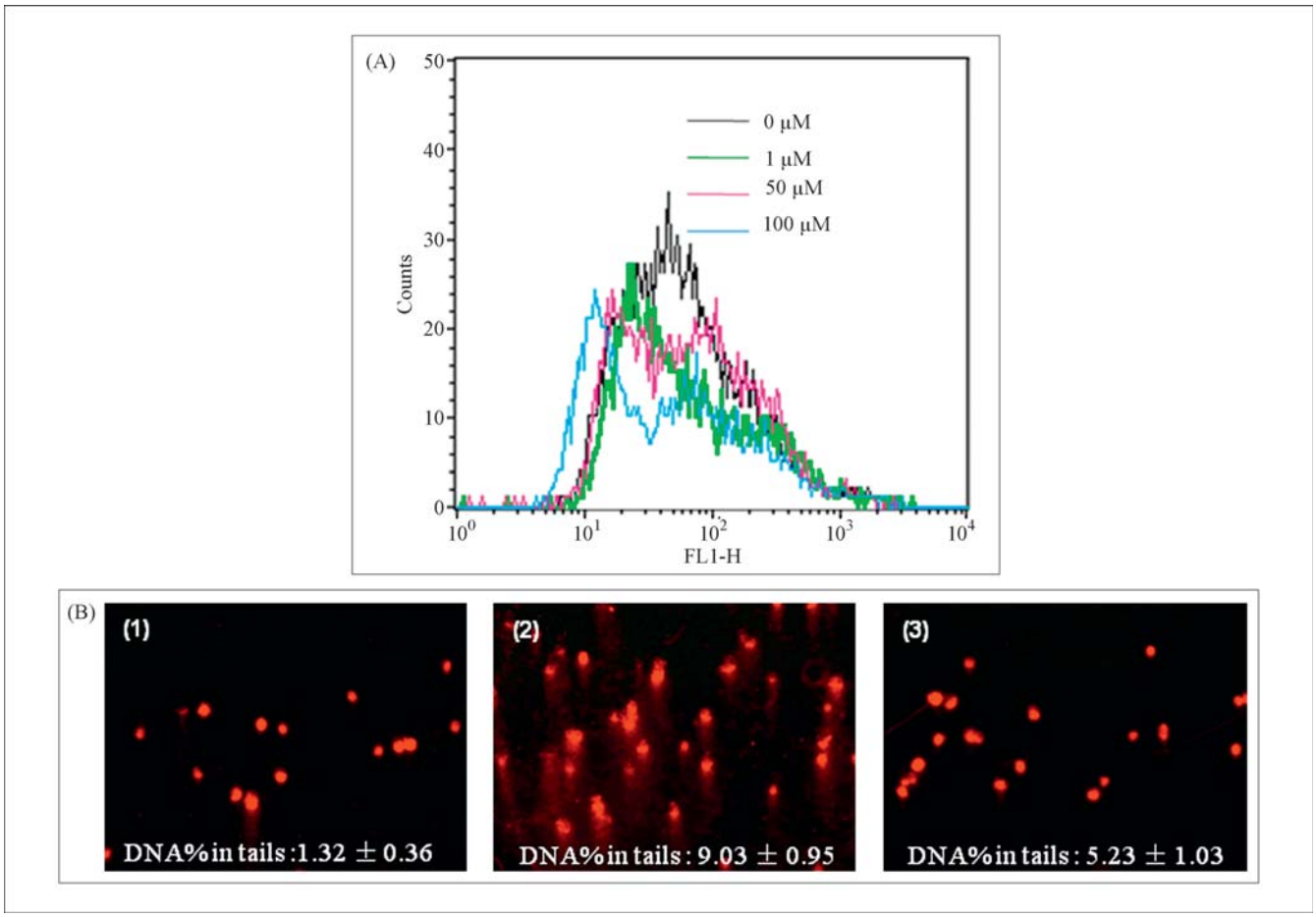

Fig. 2: Echinacoside decreases the level of intracellular ROS and prevents cells from DNA lesion
A. Echinacoside decreases the level of intracellular ROS. Different colors indicate echinacoside at concentrations of 0, 1, 50, 100 μM as labeled in the legend. B. Single cell gel electrophoresis of cells. (1) Non-treated MRC-5 cells; (2) MRC-5 cells induced with 250 μM H_2O_2; (3) MRC-5 pretreated with 100 μM echinacoside, then induced with 250 μM H_2O_2. Experiments were performed three times independently, and one typical result was presented

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