Biomedicine & Pharmacotherapy 81 (2016) 176–181

Available online at
ScienceDirect
www.sciencedirect.com

Elsevier Masson France
EM|consulte
www.em-consulte.com/en

Investigation of the anti-glioma activity of *Oviductus ranae* protein hydrolysate

Xin Sui, Xiao-hua Li, Ming-hua Duan, Ai-ling Jia, Ye Wang, Da Liu, Yi-Ping Li*, Zhi-Dong Qiu*

Changchun University of Chinese Medicine, China

ARTICLE INFO

Article history:
Received 30 November 2015
Received in revised form 6 April 2016
Accepted 7 April 2016

Keywords:
Oviductus ranae
Oviductus ranae protein hydrolysate
Glioma cells
PI3K/AKT

ABSTRACT

Oviductus Ranae is the dry oviducts of *Rana temporaria chensinensis*, and it has been reported to have a range of biological activities. This study aimed to investigate the effects of *Oviductus Ranae* protein hydrolysate (ORPH) on human glioma C_6 cell proliferation and apoptosis *in vitro* and *in vivo*. Following *in vitro* treatment, cell viability and colony formation assays showed that ORPH inhibited C_6 cell proliferation. In addition, the results of western blotting also demonstrated that ORPH effectively regulated the expression of the apoptosis related proteins, cleaved caspase-3, Bax and Bcl-2, DNA staining and flow cytometry analysis demonstrated that ORPH significantly promoted apoptosis in this cell line, a finding that was confirmed *in vivo* using terminal deoxynucleotidyl transferase dUTP nick end labeling. Further investigation demonstrated that ORPH increased apoptosis by modulating the release of inflammatory cytokines and the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway; this was demonstrated using a PI3K/AKT inhibitor (NVP-BEZ235). In summary, the present study suggested that ORPH promoted apoptosis and inhibited glioma cell proliferation by influencing the PI3 K/AKT signaling pathway.

1. Introduction

Gliomas, which originate from neuroepithelial tissue, are the most common and lethal tumors of the central nervous system, with a five-year median survival rate of less than 5% [1,2]. Histopathological and clinical criteria established by the World Health Organization showed that glioma was the IV grade that originates from poorly differentiated astrocytes [3,4]. Gliomas are characterized by rapid growth, strong invasiveness, frequent postoperative relapse, and high mortality [5]. The aggressive nature of this cancer arises from its intense cell proliferation, diffuse infiltration, and high resistance to apoptosis. Although treatment for glioma includes surgery, chemotherapy, and radiotherapy and novel approaches including gene therapy and immunotherapy are under investigation [6,7], most patients die within the first year of diagnosis [8]. To date, the molecular mechanisms underlying the tumorigenesis of gliomas are poorly understood, and this disease remains a focus of concern in the field of neurosurgery. With recent technological advancements, a number of Chinese medicines that influence the development of gliomas have been identified [9–12]. Previous studies have shown that a lot of traditional Chinese medicine have therapeutic effects on C6 glioma, such as baicalin [13], panaxydol [14], saikosaponins [9], providing new research leads relating to the diagnosis and treatment of this type of cancer.

Rana temporaria chensinensis is an amphibian that is widely distributed in northeastern China and has been used extensively in traditional oriental medicine [15–17]. The expensive unisex health product “Lin wa you” is extracted from the fallopian tube of the female *R. chensinensis*. Moreover, desiccated oviducts from female *R. chensinensis* provide a valuable Chinese crude drug known as *Oviductus Ranae* (OR), which is recorded in the Pharmacopoeia of the People’s Republic of China (2005 edition). Studies have shown that OR can replenish the kidney essence, nourish the yin, and moisten the lung [18,19]. OR also has antioxidant, anti-fatigue, and anti-inflammatory properties [20,21]. OR protein hydrolysate (ORPH) can be extracted from OR using the methods described by Huang [22]. It is also indicated that immune inflammation has an important effect on the development of glioma[23,24]. Further research is required to determine whether ORPH has the same biological effects as OR, and it’s role in glioma.

In this study, we extracted ORPH and investigated its effects on glioma cell cultures, and the underlying molecular mechanisms. The results indicated that ORPH inhibited the proliferation of

* Corresponding authors.
E-mail address: czdliyiping@163.com (Y.-P. Li).

http://dx.doi.org/10.1016/j.biopha.2016.04.015
0753-3322/

glioma cells and promoted apoptosis both *in vitro* and *in vivo*; these effects were mediated by the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway.

2. Materials and methods

2.1. Cell lines

The human glioma cell line, C_6, was purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640; Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (HyClone, Logan, UT, USA) in a humidified atmosphere with 5% CO_2 at 37 °C.

2.2. Cell treatment

Cells were divided into six groups and exposed to the treatments described below when they reached a density of 80%. The six experimental conditions were the control (Con) group, the control + NVP-BEZ235 (200 μg/ml) (Con + NB) group, the ORPH (500 μg/ml) group, the ORPH + NVP-BEZ235 (ORPH + NB) group, the cisplatin (Tumor therapy drugs) (3000 μg/ml)(Cis) group, and the ORPH + cisplatin (ORPH + Cis) group. OR were collected from the adult female Chinese brown frogs (Jilin Baekdu Mountain Chinese Brown Frog Breeding Farm, Jilin Province, China). NVP-BEZ235 (Amyjet Scientific Inc., Wuhan, China). Cisplatin (Valley of Yunnan biological pharmaceutical co., LTD, Yunnan,China).

2.3. MTT assay

Cell proliferation and viability were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well microplates at a density of 2×10^3 cells/well. The cells were incubated and allowed to adhere prior to exposure to ORPH and/or cisplatin for the indicated time periods (12, 24, 48, 72, or 96 h). MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well at a final concentration of 0.2 mg/ml and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 4 h. The supernatant was removed and formazan crystals were dissolved in 200 μl dimethyl sulfoxide (Sigma-Aldrich) for 15 min. The optical density was determined at 490 nm using a microplate reader (ELX-800; BioTek, Vermont, USA).

2.4. Colony formation assay

The treated cells were harvested using trypsin; 200 were seeded onto each 35-mm dish and incubated for 14 days at 37 °C in a humidified atmosphere with 5% CO_2. The resulting colonies were rinsed with phosphate-buffered saline (PBS), fixed with paraformaldehyde for 20 min, and stained with Giemsa (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) for 5–8 min. The number of colonies containing at least 50 cells was counted under a microscope. The colony formation efficiency was calculated as (number of colonies/number of cells inoculated) × 100%.

2.5. Hoechst33342 staining

A Hoechst staining kit (Beyotime Institute of Biotechnology, Haimen, China) was used to detect apoptotic cells, according to the manufacturer's instructions. Treated cells 1×10^4 were seeded in 12-well plates and incubated at 37 °C in a humidified atmosphere with 5% CO_2 and allowed to adhere. 70% ethanol (0.5 ml) was used to fix the cells for 20 min at room temperature. After washing with PBS, cells were stained with 0.5 ml Hoechst staining liquid for 5 min. The cells were observed under a fluorescence microscope (BX53; Olympus, Tokyo, Japan) and photographed at 400× magnification after the addition of anti-fluorescent quencher.

2.6. Flow cytometry analysis of apoptosis

Flow cytometry analysis using FACSCalibur (BD, Franklin Lakes, NJ, USA) and an apoptosis detection kit (Bioworld Technology, Nanjing, Jiangsu, China) were used to detect apoptotic cells, according to the manufacturer's instructions. Cells were harvested, centrifuged, and washed prior to resuspension in 400 μl binding buffer. Annexin V-FITC (5 μl) was added to the cell suspensions and incubated at 2–8 °C for 15 min in the dark. Propidium iodide (10 μl) was then added to the cell suspensions and incubated at 2–8 °C for 5 min in the dark, followed by flow cytometry analysis within 1 h.

2.7. In vivo experiments

Twenty-four BABL/c nude mice that weighed 18-20 g and were 4-6 weeks old were purchased from the animal center of Jilin University, China. The mice were maintained under pathogen-free conditions at 22 °C, with 40–50% humidity, a 12-h light/dark cycle, and food and water *ad libitum*. The animal care and treatment were approved by the Experimental Animal Ethics Committee of Jilin University. The mice were assigned randomly to four groups: a Con group, ORPH (1.5 g/kg, i.g.) group, Cis group (1.5 mg/kg, i.p.), and ORPH + C is group. The glioma cells (1×10^6) were suspended in 0.2 ml normal saline and then inoculated subcutaneously into the ribs on the right-hand side of the nude mice, respectively. After one week the mice were treated with different drugs as described before.

2.8. Detection of apoptosis using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

Apoptotic cells were detected using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Paraffin sections of the mouse tumor tissues were inactivated using H_2O_2 and then incubated with 50 μl TUNEL reaction solution at 37 °C for 60 min. After washing with PBS, the sections were incubated with 50 μl Converter-POD working solution at 37 °C for 30 min. The signal was developed using 3′-diaminobenzidine and the nuclei were stained using hematoxylin. The results were observed under a microscope and photographed at 400× magnification.

2.9. Western blot

NP-40 lysate buffer (Beyotime Institute of Biotechnology) was used to lyse cells and prepare total protein extracts. A cytoplasmic protein extraction kit (Beyotime) was used to prepare a nuclear protein to extract. BCA assay was used to determine the protein concentration. Each protein sample (40 μg) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transfer to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk at room temperature for 1.5 h, the membrane was incubated overnight at 4 °C with a specific primary antibody raised against PI3 K, phosphorylated (p)-PI3 K, AKT, or p-AKT (1:2000; Abcam, Shanghai, China), or cleaved caspase-3, B-cell lymphoma 2 (Bcl-2), or Bcl-2-associated X-protein (Bax), β-actin (1:500; bioss, Beijing, China). Subsequently, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (Beyotime) at 37 °C for 2.5 h. Proteins were visualized using enhanced chemiluminescence reagents (Biosharp, Anhui, China). After exposure, the film was scanned and analyzed by Gel-Pro-Analyzer