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rLj-RGD3 induces apoptosis via the mitochondrial-dependent pathway and inhibits adhesion, migration and invasion of human HeyA8 cells via FAK pathway

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ABSTRACT

Ovarian carcinoma is a tumor derived from ovary, which brings relatively higher mortality rate among the fatal gynecological cancers. Recently, lots of studies have concentrated on the anti-tumor effects of Arg-Gly-Asp (RGD) motif containing peptides due to their integrin binding properties. In order to meet the criterion of genetic engineering drugs, a recombinant RGD toxin protein (rLj-RGD3) without a His-tag was cloned from the buccal glands of *Lampetra japonica* in the present study. After endotoxin removal, the His-tag removed rLj-RGD3 was shown to inhibit the proliferation of HeyA8 cells. According to the confocal microscope, flow cytometry and western blot analysis, rLj-RGD3 could trigger HeyA8 cells apoptosis by changing mitochondrial membrane potential, arrangement of F-actin, protein level of BCL2, BAX, caspase 3, and cleaved caspase 3, concentration of cytoplasmic calcium, as well as phosphorylation level of ERK/JNK/p38. Furthermore, rLj-RGD3 was also able to suppress the adhesion, migration, and invasion processes of HeyA8 cells by disturbing the organization of F-actin and reducing the level of *p*-FAK. In addition, rLj-RGD3 could inhibit the adhesion of HeyA8 cells to extracellular matrix proteins by competitively binding to integrins, indicated that rLj-RGD3 might act as an anti-tumor drug to treat ovarian carcinoma patients in the future.

1. Introduction

In the past decades, we are not unfamiliar with the "malignant tumor" which is becoming the leading cause of death all around the world. Among the numerous tumors, ovarian carcinoma is a tumor derived from ovary, which is usually ignored by the patients due to its asymptomatic nature [1]. Once ovarian cancer cells begin to spread, pelvic pain and abdominal swelling would be diagnosed [2]. Thus, it is not surprising that ovarian carcinoma brings relatively higher mortality rate among the fatal gynecological cancers, with a five-year survival rate of approximately 45% [3,4]. In order to improve the survivability of ovarian carcinoma patients, a large number of studies have tried to figure out the molecular mechanisms and potential biomarkers which account for the development of ovarian carcinoma [3,5,6]. At present, cytoreductive surgery and adjuvant chemotherapy with platinum-based drugs are usually used in the treatment of ovarian carcinoma patients [7]. Regrettably, patients with ovarian carcinoma might suffer the adverse effects concomitant with the treatment course and might have the relatively higher tumor recurrence and metastasis rates

Abbreviations: BCA, bicinchoninic acid; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; caspase, cysteine-aspartic acid protease; DCFH-DA, 2′, 7′-dichloroflurescin- diacetate; DMEM, dulbecco's modified eagle medium; ECM, extracellular matrix; *E. coli*, *Escherichia coli*; EDTA, ethylenediaminetetraacetic acid; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high performance liquid chromatography; JNK, c-Jun N-terminal kinases; LDH, lactate dehydrogenase; *L. japonica*, *Lampetra japonica*; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MAPK, mitogen activated protein kinases; MMP, matrix metallo-proteinase; MTT, colorimetric 2 5-diphenyltetrazolium bromide; OD, optical density; ORF, open reading frame; PBS, phosphate buffered saline; PI, propidium iodide; PMSF, phenylmethanesulfonyl fluoride; PTK2, protein tyrosine kinase 2; PVDF, polyvinylidene difluoride; RGD, Arg-Gly-Asp; Rh123, rhodamine123; rLj- RGD3, recombinant *Lampetra japonica*-RGD3; ROS, reactive oxygen species; SD, Sprague Dawley; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBST, Tris buffered saline tween.

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[7]. Therefore, lots of studies are still focusing on more effective drugs to suppress the proliferation and metastasis of ovarian cancer cells.

Recently, Arg-Gly-Asp (RGD) toxins from the salivary glands of leeches, ticks, flies, mosquitoes, as well as the venom glands of snakes have been reported to play important roles in the suppression of platelet aggregation, angiogenesis, tumor cells proliferation and metastasis, due to their abilities of binding to integrins [8–14]. As the expression level of integrins in tumor cells was found increased significantly, labeled RGD had been used as a probe to target the tumors *in vivo* [15,16]. In contrast to the extensive studies of RGD toxins from the other species, little work has been done on the RGD toxins from *Lampetra japonica* (*L. japonica*). Until 2010, Wang et al. firstly identified a protein containing three RGD motifs (Lj-RGD3) in the buccal glands of *L. japonica*, which is one of the most primitive vertebrates still lived. Similar to the other RGD toxins, the recombinant *L. japonica* RGD3 (rLj-RGD3) with a His-tag was also shown not only to inhibit platelet aggregation, but also to suppress the proliferation, adhesion, migration and invasion of ECV304 cells [17]. 2012, Jin et al. reported that rLj-RGD3 was more effective on inhibition the proliferation of drug-resistant MCF-7/Adr breast carcinoma cells than on the drug-sensitive MCF-7 cells [18]. In addition, rLj-RGD3 was also able to suppress TNF-α-induced proliferation, migration and invasion of human renal carcinoma cells by down-regulating the protein level of matrix metallo-proteinase 9 (MMP-9) [19]. Although rLj-RGD3 was found to induce apoptosis in ECV304, breast carcinoma and human renal carcinoma cells, the detailed mechanisms have not been discussed in the previous studies.

Although rLj-RGD3 inhibited the proliferation of ECV304, breast and renal cancer cells, could rLj-RGD3 also work in ovarian carcinoma? In the present study, the His-tag was removed from rLj-RGD3 in order to meet the criterion of genetic engineering drugs. And the effects of the His-tag removed rLj-RGD3 on ovarian cancer cells (HeyA8 cells) were analyzed and the apoptotic mechanisms and related signal pathways were also discussed in detail.

2. Materials and methods

2.1. Preparation of His-tag removed rLj-RGD3

According to the previous study reported by Wang et al., a termination codon was added into the end of the open reading frame (ORF) sequence of rLj-RGD3 [17]. The primers with Nde *I* and Hind *III* restriction enzyme sites are designed as follows: 5′-catatgtcaacgttcatcaacggaacc-3′; 5′-aagctttcactccccaacacattcact−3′. Subsequently, rLj-RGD3 was subcloned into a pET23b vector and expressed as a soluble protein without a His-tag. Due to the high content of histidine residues, rLj-RGD3 with the molecular weight of 13.5 kDa was purified by a Nickel-column (GE Healthcare, USA). Subsequently, the endotoxin in the purified rLj-RGD3 was removed by separation on a Q anion exchange chromatography (GE Healthcare, USA). The content of endotoxin was determined by Tachypleus Amebocyte Lysate For endotoxin (Pyrogen) Detection Kit (Chinese Horseshoe Crab Reagent Manufactory Co., Ltd) according to the manufacturer's instructions. After dialysis and lyophilization, the purified protein was electrophoresed on reducing Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by high performance liquid chromatography (HPLC) which was packed with TSKgel G3000swXL and eluted with buffer containing 3.2 mM Na_2HPO_3, 1.5 mM KH_2PO_3 and 400 mM NaCl (pH 7.3). The N-terminal amino acid sequences of the purified protein were analyzed by Edman degradation on an automated protein sequencer (Procise 491, USA). And the molecular mass of the purified rLj-RGD3 was detected by a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS, Bruker, USA). Finally, the rLj-RGD3 concentration was determined using a bicinchoninic acid (BCA) protein assay Kit (Beyotime Biotechnology, China) with bovine serum albumin (BSA) as a standard.

2.2. Cell culture

HeyA8 ovarian cells were obtained from Prof. Pixu Liu in Dalian Medical University. The HeyA8 cells were cultured in dulbecco's modified eagle medium (DMEM, GIBCO, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS, GIBCO) in a humidified incubator with 5% CO_2 at 37 °C.

2.3. MTT and lactate dehydrogenase (LDH) assays

HeyA8 cells were allowed to grow on the 96-well plates in DMEMsupplemented with 10% FBS. After removing the medium, the HeyA8 cells were treated with phosphate buffered saline (PBS) or different concentrations of rLj-RGD3 (from 0.8 μM to 51.2 μM) for 24 h. The proliferation rate of HeyA8 cells was detected by the classic colorimetric 2, 5-diphenyltetrazolium bromide (MTT) assay. The PBS treated HeyA8 cells were defined as the control group and their absorbance at 492 nm (optical density value, OD value) was described as 100%. The proliferation rate of HeyA8 cells in the rLj-RGD3 treating groups was calculated according to the formula below: the proliferation rate of HeyA8 cells (% of control) = rLj-RGD3 treating groups' OD value/PBS treating group's OD value × 100%. In addition, the cytotoxicity of rLj-RGD3 on HeyA8 cells was measured using the LDH assay Kit (Beyotime Biotechnology, China). The HeyA8 cells were also treated with PBS or rLj-RGD3 with the same concentrations as MTT assay. Subsequently, the LDH activity in the DMEM of HeyA8 cells was monitored by a microplate reader (Thermo SCIENTIFIC, USA). A LDH release factor was used as a positive control and its absorbance at 492 nm (OD value) was defined as 100%. The cytotoxicity was calculated according to the formula below: the cytotoxicity (% of control) = PBS or rLj-RGD3 treating groups' OD value/LDH release factor treating group's OD value × 100%.

2.4. The fluorescent staining assay

The HeyA8 cells (1×10^5) were cultured on slides in 24-well plates for 24 h, and then treated with PBS, 10 μM, 15 μM or 20 μM rLj-RGD3 for 12 h. After rinsing with PBS, the HeyA8 cells were fixed with 4% paraformaldehyde for 10 min. Subsequently, the HeyA8 cells were washed with PBS twice, and then stained with FITC labeled phalloidin (Enzo Life Sciences, Swiss) for 15 min at room temperature in the dark. In order to remove any traces of non-specific fluorescence, the cells were washed with PBS twice, and then stained with Hoechst 33258 (Beyotime Biotechnology, China) for 5 min at room temperature in the dark. After washing with PBS twice, a laser scanning confocal microscopy (Carl Zeiss, Germany) was used to observe the F-actin and cell nucleus at 630 × magnification.

2.5. Flow cytometry assay

The HeyA8 cells were grown on the 6-well plates for 24 h and then treated with PBS, 10 μM, 15 μM or 20 μM rLj-RGD3 for another 24 h. After removing the medium, the adherent HeyA8 cells were digested with ethylenediaminetetraacetic acid (EDTA) free trypsin (HyClone, USA), collected through centrifugation and resuspended in PBS buffer. As for the Annexin V/propidium iodide (PI) double staining assay, the HeyA8 cells were incubated with