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Polygonatum odoratum lectin induces apoptosis and autophagy via targeting EGFR-mediated Ras-Raf-MEK-ERK pathway in human MCF-7 breast cancer cells

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ABSTRACT

Polygonatum odoratum lectin (POL), a mannose-binding GNA-related lectin, has been reported to display remarkable anti-proliferative and apoptosis-inducing activities toward a variety of cancer cells; however, the precise molecular mechanisms by which POL induces cancer cell death are still elusive. In the current study, we found that POL could induce both apoptosis and autophagy in human MCF-7 breast cancer cells. Subsequently, we found that POL induced MCF-7 cell apoptosis via the mitochondrial pathway. Additionally, we also found that POL induces MCF-7 cell apoptosis via EGFR-mediated Ras-Raf-MEK-ERK pathway, suggesting that POL may be a potential EGFR inhibitor. Finally, we used proteomics analyses for exploring more possible POL-induced pathways with EGFR, Ras, Raf, MEK and ERK, some of which were consistent with our in silico network prediction. Taken together, these results demonstrate that POL induces MCF-7 cell apoptosis and autophagy via targeting EGFR-mediated Ras-Raf-MEK-ERK signaling pathway, which would provide a new clue for exploiting POL as a potential anti-neoplastic drug for future cancer therapy.

Introduction

Plant lectins are a group of highly diverse non-immune origin proteins ubiquitously distributed in a variety of plant species and they contain at least one non-catalytic domain that enables them to selectively recognize and reversibly bind to specific free sugars or glycans present on glycoproteins and glycolipids without altering the structure of the carbohydrate (Van Damme et al., 1998). Of note, plant lectins can be divided into 12 different families, including ABA (*Agaricus bisporus* agglutinin), Amaranthin, CRA (chitinase-related agglutinin), Cyanovirin, EEA (*Euonymus europaeus* agglutinin), GNA (*Galanthus nivalis* agglutinin), Hevein, Jacalins, Legume lectin, LysM (lysin motif), Nictaba and Ricin_B families (Van Damme et al., 2008). Amongst the above-mentioned families, *Galanthus nivalis* agglutinin (GNA)-related lectins have been reported to possess a broad range of biological activities, such as anti-tumor activities (Tian et al., 2008; Van Damme et al., 2007; Li et al., 2009).

In the previous studies, several GNA-related lectins, such as *Polygonatum cyrtonema* lectin (PCL), *Ophiopogon japonicus* lectin (OJL) and *Liparis noversa* lectin (LNL), were reported to possess remarkable anti-proliferative and apoptosis-inducing activities toward various cancer cells (Liu et al., 2009a,2009b,2009c,2009d; Wang et al., 2011). Moreover, *Polygonatum odoratum* lectin (POL), isolated from crude extracts from rhizomes of polygonatum odoratum (Mill.), is a homo-tetramer with molecular weight of 11,953.623 Da per subunits as purified by gel filtration, SDS-PAGE, and determined by mass spectrometry. And, POL bears three conserved motif of 'QXDXNXVXY', which is essential in the mannose recognition, and it can agglutinate rabbit erythrocytes at a minimal concentration (≥3.75 μg/ml) (Yang et al., 2011). Interestingly, *Polygonatum odoratum* lectin (POL) has been recently reported to induce apoptosis via death-receptor and mitochondrial apoptotic pathways in murine fibrosarcoma L929 cells (Liu et al.,

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2009a,2009b,2009c,2009d; Fu et al., 2011; Yu et al., 2011). However, the precise mechanisms by which POL induces cancer cell death are still rudimentarily understood.

In this study, we report for the first time that POL induces mitochondrial apoptosis and autophagic cell death via targeting EGFR-mediated Ras-Raf-MEK-ERK signaling pathways in MCF-7 cells. These results may provide new evidence for further understanding key apoptotic and autophagic pathways induced by such GNA-related lectin for future cancer drug discovery.

Materials and methods

Reagents

Polygonatum cyrtonema lectin (POL) was purified as previously described. The purification was first applied to a CM-Sepharose column and eluted with 0 – 0.5 M NaCl gradient in 40 mM, NaAc – HAc buffer (pH 4.6) and peak fractions with hemagglutinating activity were collected. Then the pooled active fractions were concentrated and loaded on the column of Sephacyl S-100. Finally, the peak fraction showing hemagglutinating activity was purified POL (Yang et al., 2011). Fetal bovine serum (FBS) was purchased from TBD Biotechnology Development (Tianjin, China); 3-(4,5-dimetrylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3,3-diaminobenzidine tetrahydrochloride (DAB), and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, MO, USA). Rabbit polyclonal antibodies against EGFR, Ras, Raf, MEK, ERK, LC3, Beclin-1, β-actin, and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

The MCF-7 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.03% L-glutamine and maintained at 37 °C with 5% CO_2 at a humidified atmosphere. All the experiments were performed on logarithmically growing cells.

Growth inhibition assay

The cytotoxic effect of POL in MCF-7 cells was measured by the MTT assay as described elsewhere (Cheng et al., 2010). The MCF-7 cells were incubated in 96-well tissue culture plates (NUNU, Roskilde, Denmark) at a density of 5×10^4 cells/well. The cytotoxic effect was measured with a plate reader by the MTT assay. The percentage of cell growth inhibition was calculated as follows: Cell growth inhibition (%) = $[A_{492}\,(\text{control}) - A_{492}\,(\text{POL})]/[A_{492}\,(\text{control}) - A_{492}\,(\text{blank})] \times 100$.

Apoptosis assay

Human breast adenocarcinoma MCF-7 cells were seeded into 96-well culture plates with or without POL and cultured for 24 h. The ultrastructure of cell apoptosis was observed under the electron microscope (Hitachi7000, Japan) (Cheng et al., 2008). The collected cells were fixed with 500 μl PBS and 10 ml 70% ethanol at 4 °C overnight; then after washing twice with PBS, the cells were incubated with 1 ml Hoechst staining solution for 30 min at 4 °C. The percentage of cells at different phases of the Sub-G1 DNA content was measured by flow cytometry (Becton Dickinson, Franklin Lakes, NJ).

Autophagy assay

After incubation with POL for the fixed times, the MCF-7 cells were cultured with 0.05 mM MDC at 37 °C for 60 min. The fluorescence intensity of cells was analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ) (Cheng et al., 2009a,2009b). Cells were transfected with GFP-LC3 plasmid (kindly provided by Prof. Canhua Huang, Sichuan University) using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The fluorescence of GFP-LC3 was observed under a fluorescence microscope.

Small interfering RNA (siRNA) transfection

siRNA against human EGFR and control siRNA was purchased from Invitrogen (Carlsbad, CA). Cells were transfected with siRNAs at 33 nM final concentration using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The transfected cells were used for subsequent experiments 24 h later.

Western blot analysis

The MCF-7 cells were treated with 10 μg/ml POL for 6, 12, 18 and 24 h, respectively. Both adherent and floating cells were collected, and then Western blot analysis was carried out as previously described (Cheng et al., 2009a,2009b). Briefly, the cell pellets were resuspended with lysis buffer consisting of Hepes 50 mmol/l pH 7.4, Triton-X-100 1%, sodium orthovanada 2 mmol/l, sodium fluoride 100 mmol/l, edetic acid 1 mmol/l, PMSF 1 mmol/l, aprotinin (Sigma, MO, USA) 10 mg/l and leupeptin (Sigma) 10 mg/l and lysed at 4 °C for 1 h. After 12,000*g* centrifugation for 15 min, the protein content of supernatant was determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of the total protein were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes, the membranes were soaked in blocking buffer (5% skimmed milk). Proteins were detected using polyclonal antibodies and visualized using anti-rabbit or anti-mouse IgG conjugated with peroxidase (HRP) and 3,3-diaminobenzidine tetrahydrochloride (DAB) as the HRP substrate.

2-DE and MS/MS analysis

2-DE and MS/MS analysis were performed as described previously (Zhang et al., 2013). Briefly, cells were dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 0.2% pH 3 – 10 ampholyte, Bio-Rad, USA) in presence of protease inhibitor (Sigma). Samples were loaded into IPG strips (17 cm, pH 3 – 10 NL, Bio-Rad) using a passive rehydration method, and then subjected to isoelectric focusing (Bio-Rad). The second dimension separation was performed using 12% SDS-PAGE after equilibration. The gels were stained with CBB R-250 (Bio-Rad). Identification and quantitation of protein spots in a gel was performed by using PDQuest software (Bio-Rad). In-gel protein digestion was performed using mass spectrometry grade trypsin according to the manufacturer's instructions. The gel spots were destained with 100 mM NH_4HCO_3/50% acetonitrile (ACN) and dehydrated with 100% ACN. The gels were then incubated with trypsin (Promega, V5280), followed by double extraction with 50% ACN/5% trifluoroacetic acid (TFA). The peptide extracts were dried in a speed-VAC concentrator (Thermo), and subjected to mass spectrometric analysis using a Q-TOF mass spectrometer (Micromass, Manchester, UK) fitted with an ESI source.