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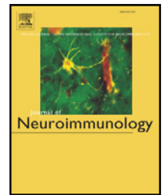

Parthenolide inhibits the initiation of experimental autoimmune neuritis

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ABSTRACT

A growing body of evidence suggests the anti-inflammatory and antitumor effects of parthenolide (PAR). Here we show that PAR treatment inhibits the initiation of experimental autoimmune neuritis (EAN), suppresses the production of TNF-α, IFN-γ, IL-1β and IL-17, and decreases Th1 and Th17 cells at early time point. However, such anti-inflammatory effect vanishes later and PAR impedes the recovery of EAN in late phase, which is accompanied with inhibited apoptosis of inflammatory cells. Our results indicate that PAR plays dual roles in EAN and it is not proper to be applied in autoimmune diseases of nervous system.

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1. Introduction

Feverfew (*Tanacetum parthenium* L.) has been used as a medicinal herb for centuries in Europe to treat a variety of diseases, including arthritis, fever, headache, psoriasis, menstrual disorders, inflammatory conditions, etc. Its name originates from the Latin word *febrifugia*, which means fever alleviator. Parthenolide (PAR) is a sesquiterpene lactone, which has been identified as the principle active component of feverfew (Pareek et al., 2011). PAR has been proved to inhibit NF-κB activation and it is often sold as a NF-κB inhibitor (Ghantous et al., 2013). Besides, PAR was shown to directly inhibit the enzyme activity of caspase-1 and inhibit the activation of inflammasomes (Juliana et al., 2010). Its anti-inflammatory functions have been documented in various animal models such as cyclophosphamide-induced cystitis, sulfate sodium-induced colitis and collagen-induced arthritis (Kiuchi et al., 2009; Zhao et al., 2012; Liu et al., 2015). Moreover, PAR was shown to induce apoptosis of cancer cells and selectively inhibit cancer stem cells while sparing normal stem cells. The antitumor effects of PAR and its hydrophilic derivative have been proved in a number of cell and animal experiments (Ghantous et al., 2013).

Guillain–Barré syndrome (GBS) is an acute inflammatory disease targeting peripheral nervous system. It is characterized by symmetrical muscle weakness with rapid aggravation. The exact causes of GBS are not clear and effective treatments are limited (Esposito and Longo, 2017). Experimental autoimmune neuritis (EAN) is an animal model for GBS, just as experimental autoimmune encephalitis (EAE) for multiple sclerosis. EAN could be induced in susceptible animal species with peripheral nerve tissue homogenates, myelin proteins or synthetic peptides of myelin proteins. EAN has been widely used to study the pathogenesis of GBS and to explore new therapies (Gold et al., 2000; Zhang et al., 2013).

Although PAR has exhibited anti-inflammatory effects in various cells and animal models, its role in autoimmune diseases of nervous system has not been reported. To evaluate the effects of PAR in immune response in EAN, Lewis rats were immunized with bovine peripheral myelin (BPM) and treated with different doses of PAR. Inflammatory cytokine production and $CD4^{+}$ T cell populations were analyzed both in early phase (just before symptoms appeared) and in late phase (when the symptoms peaked) of EAN. Besides, the effects of PAR on lymphocyte proliferation and proinflammatory cytokine production *in vitro* were also tested.

2. Materials and methods

2.1. Animals and reagents

Lewis rats (body weight, 180–230 g) were purchased from Vital River Corporation (Beijing, China). The rats were allowed to acclimate for one week before the experiments. All rats were housed in local specific pathogen-free animal facility with free access to food and water.

Abbreviations: PAR, parthenolide; EAN, experimental autoimmune neuritis; GBS, Guillain–Barré syndrome; EAE, experimental autoimmune encephalomyelitis; BPM, bovine peripheral myelin; MNCs, mononuclear cells; i.p., intraperitoneal; p.i., post-immunization; CFSE, carboxy-fluorescein diacetate, succinimidyl ester; FBS, fetal bovine serum; ConA, Concanavalin A; HE, hematoxylin and eosin; MFI, mean fluorescence intensity; IL-17, IL-17A; PI., Propidium Iodide.

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The experiment protocols have been approved by the institutional ethics committee.

Bovine peripheral myelin (BPM) was prepared according to previous report (Norton and Poduslo, 1973). Parthenolide (PAR) was purchased from Selleck Chemicals (Shanghai, China).

2.2. Induction of EAN and assessment of clinical symptoms

Rats were immunized by subcutaneously injection of 200 μl inoculum in the base of tail. The inoculum was composed of 1 mg BPM and 0.3 mg *Mycobacterium tuberculosis* (strain H37RA; Difco, Detoit, MI, USA), which were emulsified in 100 μl incomplete Freud adjuvant (Sigma-Aldrich, USA) and 100 μl saline.

The rats were observed and assessed daily after immunization. Clinical symptoms were graded as follows: 0 = no illness; 1 = flaccid tail; 2 = ataxia or mild paraparesis; 3 = moderate paraparesis; 4 = severe paraparesis; 5 = tetraparesis; 6 = moribund; 7 = death.

2.3. Treatment with PAR

The rats were randomly divided into three groups: control group, low dose treatment group with 2 mg/kg PAR and high dose treatment group with 8 mg/kg PAR. The treatments started on day 5 post immunization (p.i.) when the immune response *in vivo* was established (De Silva and Klein, 2015). PAR was dissolved in 100 μl dimethyl sulfoxide and given to rats *via* intraperitoneal (i.p.) injection. The rats in control group received the same volume of solvent in the same way. Rats were treated daily until the end of experiments.

The doses of PAR and the treatment regimens were determined based on previous reports (Zhao et al., 2012; Wang et al., 2016; Zhao et al., 2016; Xie et al., 2012). According to our preliminary experiments, Lewis rats induced with BPM showed symptoms of paralysis after day 10 p.i. The symptoms progressed rapidly and peaked around day 14 p.i. Rats from control group recovered gradually after day 14 p.i. Thus, the two time points, day 10 p.i. and day 14 p.i., were chosen to explore the effects of PAR on EAN. On day 10 p.i., just before the signs of weakness appeared, one set of rats (4 rats in each group) was sacrificed after anesthetization, and on day 14 p.i., when symptoms peaked, another set of rats was sacrificed. Serum, inguinal lymph nodes and sciatic nerves of each rat were harvested for further examination.

2.4. Analysis of proinflammatory cytokine production and CD4^{+} T lymphocyte populations of lymph node mononuclear cells (MNCs) by flow cytometry

Lymph nodes were grained through 70 μm cell strainers to get lymph node MNCs. The MNCs were suspended in RPMI 1640 medium (Hyclone, Beijing, China) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, grand land, NY, USA) and 1% (v/v) penicillin-streptomycin (Hycolone, Logan, UT, USA).

Before staining, MNCs from each rat were incubated in the presence of cell stimulation cocktail plus protein transport inhibitors (eBioscience, USA) for 5 h in the incubator at 37 °C according to the product instruction. After incubation, MNCs were collected and washed with flow cytometry binding buffer. Cell surface antigen CD4 was first stained with FITC conjugated anti-rat CD4 antibody (eBioscience, USA). Then cells were fixed with 2% paraformaldehyde, permeabilized with permeabilization wash buffer (Biolegend, USA), and stained with PE anti-TNF-α (Biolegend, USA), APC anti-IL-17A (eBioscience, USA) or PE anti-IFN-γ (Biolegend, USA) antibody respectively. All the procedures were carried out according to the reagent instructions. After staining, cells were analyzed with a flow cytometer.

For the transcription factor Foxp3 detection, MNCs from each rat were first stained with surface antibody FITC anti-CD4 (eBioscience, USA), then cells were fixed and permeabilized with Foxp3 staining buffer set (eBioscience, USA) according to the instruction. After permeabilization, cells were stained with PE anti-Foxp3 antibody (eBioscience, USA). Finally, cells were washed with permeabilization buffer and analyzed with a flow cytometer.

2.5. Detecting of apoptosis by flow cytometry

The apoptosis of MNCs were detected with the FITC Annexin V Apoptosis Detection Kit with Propidium Iodide Solution (PI) (Biolegend, USA). Briefly, MNCs harvested from lymph nodes as mentioned above were washed twice with Annexin V binding buffer, then MNCs were incubated with FITC Annexin V and PI according to the instruction. After incubation, cells were suspended in Annexin V binding buffer and analyzed with a flow cytometer.

2.6. Analysis of the levels of IL-1β in sera by ELISA

Sera harvested from rats were stored at −20 °C before examination. The levels of IL-1β in sera were examined with rat IL-1β ELISA kit (Dakewe Bioengineering, Beijing) according to the instruction manual.

2.7. Histological analysis of sciatic nerves

To evaluate the infiltration of inflammatory cells in peripheral nerve system, sciatic nerves were collected and fixed with 4% paraformaldehyde. After dehydration, the nerves were embedded in paraffin and sliced into sections (3 μm). After hematoxylin and eosin (HE) staining, sections were observed under a microscope.

2.8. Lymphocyte proliferation assay in vitro

Lymphocyte proliferation assay *in vitro* was performed to explore the direct effects of PAR on lymphocytes. Inguinal lymph nodes were harvested from EAN rat on day 8 p.i. Lymph nodes were grained as mentioned above and MNCs were collected. MNCs (1×10^7 cells/ml) suspended in RPMI 1640 were stained with carboxy-fluorescein diacetate, succinimidyl ester (CFSE, 2.5 μM, Invitrogen, UK) at 37 °C for 15 min. Then cells were washed three times with complete culture medium (RPMI 1640 supplemented with 10% FBS and 1% penicillin-streptomycin). Stained MNCs (2.5×10^6 cells/ml) were planted in 96 well plates in triplicates (200 μl/well). Lymphocyte proliferation was stimulated with Concanavalin A (ConA, 2.5 μg/ml, Sigma-Aldrich, USA) or BPM (10 μg/ml) in the presence of a series of concentrations of PAR (0 μM, 1 μM, 2.5 μM and 5 μM). The concentrations of PAR were determined according to preliminary experiments and previous reports (Cavallini et al., 2001; Ku and Lin, 2013). Cells were cultured in the incubator at 37 °C. 72 h later the culture supernatants were collected for cytokine detection. Cells were collected, washed and analyzed with a flow cytometer.

The levels of IL-17A (IL-17) and TNF-α in culture supernatants were analyzed with IL-17 and TNF-α ELISA kits (eBioscience, USA) according to the instruction manuals. Results were processed and expressed in pg/ml.

2.9. Statistical analysis

Statistical analysis was performed with GraphPad Prism 6. Clinical scores were analyzed by nonparametric test. The other data were analyzed by ANOVA. Data were presented in mean ± SD. The significance level was set at $p < 0.05$.

3. Results

3.1. PAR inhibits lymphocyte proliferation in vitro

To evaluate the effects of PAR on lymphocytes, lymphocyte proliferation assay *in vitro* was performed. CFSE could diffuse into cells and

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