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Original article

Physalin F, a seco-steroid from *Physalis angulata* L., has immunosuppressive activity in peripheral blood mononuclear cells from patients with HTLV1-associated myelopathy

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ABSTRACT

Human T-lymphotropic virus type 1 (HTLV-1) induces a strong activation of the immune system, especially in individuals with HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Physalin F is a secosteroid with potent anti-inflammatory and immunomodulatory activities. The present study aimed to investigate the effects of physalin F on peripheral blood mononuclear cells (PBMC) of HAM/TSP subjects. A concentration-dependent inhibition of spontaneous proliferation of PBMC from HAM/TSP subjects was observed in the presence of physalin F, as evaluated by ^{3}H-thymidine uptake. The IC_{50} for physalin F was 0.97 ± 0.11 μM. Flow cytometry analysis using Cytometric Bead Array (CBA) showed that physalin F (10 μM) significantly reduced the levels of IL-2, IL-6, IL-10, TNF-α and IFN-γ, but not IL-17A, in supernatants of PBMC cultures. Next, apoptosis induction was addressed by using flow cytometry to evaluate annexin V expression. Treatment with physalin F (10 μM) increased the apoptotic population of PBMC in HAM/TSP subjects. Transmission electron microscopy analysis of PBMC showed that physalin F induced ultrastructural changes, such as pyknotic nuclei, damaged mitochondria, enhanced autophagic vacuole formation, and the presence of myelin-like figures. In conclusion, physalin F induces apoptosis of PBMC, decreasing the spontaneous proliferation and cytokine production caused by HTLV-1 infection.

1. Introduction

Human T-lymphotropic virus type 1 (HTLV-1) infects 5–10 million people, mainly in Latin America, the Caribbean, South and Central Africa, and Japan [1]. The virus is the etiological agent of two major diseases, adult T-cell leukemia and lymphoma (ATL) and a progressive neurologic disease, known as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), which occur in less than 5% of infected individuals[2,3]. Less frequently, the virus causes uveitis and infective dermatitis [4,5]. In addition, HTLV-1-infected individuals are more prone to other infectious diseases such as disseminated strongyloidiasis, severe scabies, and tuberculosis, suggesting an impairment in the immune response [6–10].

HTLV-1 preferentially infects memory CD45RO^{+} CD4^{+} and CD8^{+} T-lymphocytes, monocytes, and dendritic cells, leading to persistent infection and strong immune activation [11–13]. Spontaneous proliferation of CD4^{+} and CD8^{+} T-cell subsets, as well as NK cells, are found in HTLV-1-infected individuals in both in vitro and in vivo [14–17]. T-lymphocyte activation, reduced lymphoproliferative response to recall antigens in vitro and high production of proinflammatory cytokines such as IFN-γ, TNF-α, IL-2, IL-6 and IL-10 are mainly reported in patients with HAM/TSP [15,18,19]. It has been proposed that immune activation and increased levels of cytokines play a role in both pathology and progression towards HAM/TSP [18–20].

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Since HAM/TSP was first described, little progress has been made in the development of treatment options. Due to their anti-inflammatory properties, steroids are widely used for the treatment of HAM/TSP, although few benefits have been observed [21]. *Physalis angulata* L. (Solanaceae) is a broadly distributed indigenous herb found in areas of Africa, Asia, and the Americas. It is widely used in popular medicine because of its analgesic, anti-inflammatory, and antirheumatic properties [22]. Physalins are steroid derivatives isolated from *Physalis* spp. with potent anti-inflammatory and immunomodulatory activities [22–26]. Physalin F prevents mortality induced by lethal injection of lipopolysaccharide (LPS), and inhibits rejection of allogeneic transplants in mice [22–24]. The anti-inflammatory activity of physalin F was also demonstrated in intestinal ischemia, reperfusion injury, and arthritis models [27,28]. In addition, physalin F inhibits the production of TNF-α, IL-6, IL-12, and of NF-kB, a key inflammatory transcription factor [23,26]. The purpose of this study was to investigate the immunomodulatory effects of physalin F on peripheral blood mononuclear cells (PBMC) obtained from subjects with HAM/TSP. Given the immunopathological mechanisms of HTLV-1 infection, the effects of physalin F were evaluated on spontaneous cell proliferation, cytokine profile, apoptosis, and ultrastructural changes of PBMC.

2. Material and methods

2.1. Subjects

Twenty one HTLV-1-infected subjects with HAM/TSP diagnosis defined according to World Health Organization criteria followed at Bahiana School of Medicine and Public Health reference center for HTLV in Salvador, Northeast Brazil were included in the study [29]. Samples were screened for HTLV-1/2 antibodies, using enzyme-linked immunosorbent assay (Ab-Capture ELISA test system; Ortho-Clinical Diagnostics, Inc., Raritan, NJ) and confirmed by using western blotting (HTLV Blot 2.4; Genelabs Technologies, Singapore). The group had a mean age of 61 years and consisted of 14 women (67%) and 7 men (33%). Informed consent was obtained from all enrolled subjects, and the Institutional research boarding of the Oswaldo Cruz Foundation (FIOCRUZ) approved this study (Protocol 1.011.669.)

2.2. Culture conditions and PBMC isolation

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized venous blood samples by Ficoll-Hypaque density gradient centrifugation (Pharmacia Biotech; Uppsala, Sweden). Cells were cultured in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MD) supplemented with 2 mM L-glutamine (Sigma–Aldrich), 1% nonessential amino acids (Gibco Laboratories, Gaithersburg, MD), 1 mM sodium pyruvate (Sigma–Aldrich), 100 U/mL penicillin (Sigma–Aldrich), 100 μg/mL streptomycin (Sigma–Aldrich), 100 μg/mL HEPES (Invitrogen, Eugene, OR), and 10% fetal bovine serum (FBS; Gibco Laboratories).

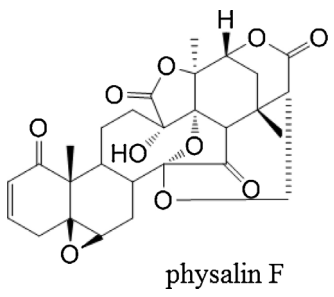

Fig. 1. Chemical structure of physalin F isolated from *P. angulata*.

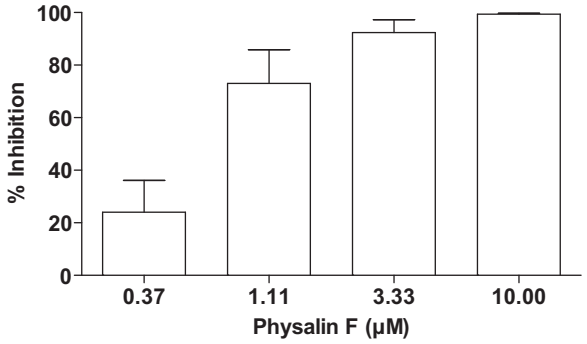

Fig. 2. In vitro inhibition of spontaneous proliferation of PBMC from subjects with HAM/TSP by physalin F. PBMC isolated from three patients with HAM/TSP diagnosis were cultured in the absence or presence of different concentrations of **1** for three days. Cell proliferation was measured by ^{3}H-thymidine incorporation using a β-radiation counter. Values represent the means ± SEM of three patients.

2.3. Test substance

Physalin F was isolated from *Physalis angulata* L. collected in Bele'm do Para', Brazil, as described previously [22]. Preparation of **1** (97.8% purity by HPLC) was dissolved in DMSO (Sigma–Aldrich) and then diluted in cell culture medium. The final concentration of DMSO was below 1% in all experiments.

2.4. In vitro cellular toxicity assay

PBMC (10^5 cells/well) from HTLV-infected patients and uninfected controls were cultured in 96-well plates in the absence or presence of serial dilutions of **1** (ranging from 0.62 to 20 μM) at 37 °C in a 5% CO_2 humidified atmosphere. After 24 h of culture, cells were pulsed for three h with 20 μL of 5 mg/mL MTT (3-[4,5-Dimethylthiazol 2yl]-2,5 diphenyltetrazolium bromide; Thiazolyl blue; Sigma–Aldrich). The optical density (OD) was determined by Versamax photometer (Molecular Devices Inc., Menlo Park, CA) at 570 nm. The toxicity was evaluated by the ratio of OD of a well in the presence of physalin F with the OD of control wells in the presence of medium. Concentrations associated to cellular viabilities equal or greater than 80% were considered non-toxic.

2.5. Lymphoproliferation assay

PBMC (10^5 cells/well) from three subjects with HAM/TSP diagnosis were cultured in the absence or presence of different concentrations of physalin F on RPMI 1640 medium supplemented with 10% FBS. Cells were seeded in triplicate on 96-well plates and cultured at 37 °C in a 5% CO_2 humidified atmosphere for 72 h. A 1 μCi/well amount of [methyl-^{3}H]thymidine (PerkinElmer, Waltham, MA) was added to the cultures, which were then incubated for 18 h at 37 °C and 5% CO_2. After this period, the content of the plate was harvested to determine the ^{3}H-thymidine incorporation using a β-radiation counter (Chameleon, Hydex; Turku, Finland). Results of cell proliferation were expressed as mean counts per minute.