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Effects of gallic acid on signaling kinases in murine macrophages and immune modulation against *Brucella abortus* 544 infection in mice

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ABSTRACT

In this study, we investigated the effects of gallic acid (GA) in intracellular signaling within murine macrophages and its contribution to host immunity during *Brucella* infection. *In vitro* analysis revealed that GA treatment decreased F-actin content and suppressed p38α phosphorylation level. *In vivo* analysis showed that GA treatment reduced inflammation and proliferation of *Brucella* in spleens of mice in comparison to PBS treatment yielding a significant protection unit. For the analysis of immune response, the uninfected GA-treated mice showed increased production of IFN-γ and MCP-1, and the *Brucella*-infected GA-treated mice showed elevated levels of IL-12p70, TNF, IFN-γ, MCP-1, IL-10 and IL-6 in comparison to negative and positive control groups, respectively. These findings demonstrate the therapeutic effects of GA against *Brucella* infection through interference on intracellular signaling pathway, induction of cytokine production and protection from bacterial proliferation in spleens of mice.

1. Introduction

Brucella species is a facultative intracellular pathogen which causes a relapsing fever and flu-like symptoms with multiple organ involvement in humans, and induces abortion and sterility in domestic animals [1]. This pathogen naturally infects a variety of wild and domestic animals, and human brucellosis represents the most common chronic zoonosis worldwide [2]. As a stealthy pathogen, *Brucella* evades innate immunity, barely activates polymorphonuclear neutrophils (PMNs), and resists the killing mechanisms of these phagocytes [3]. Microbial adhesion to host cells triggers invasion that requires actin polymerization and activation of mitogen-activated protein kinases (MAPKs) which play an important role in the phagocytosis and actin cytoskeleton remodeling [4–6].

Gallic acid (GA) is one of the main polyphenolic compounds in green tea, black tea, grapes, mango, areca nut, walnut, different berries and other plants and fruits as well as wine [7,8]. GA has been reported to have potent free radical scavenging, anti-oxidative activities and a variety of different pharmacological activities including anti-inflammatory, anti-obesity and anti-cancer activities [9]. The tannin components of *Galla Rhois* (GR) which include GA, apparently showed anti-microbial properties against *Clostridium perfringens, Escherichia* (*E.*) *coli* and *B. abortus* infection [10,11]. In addition, GA is known to inhibit the growth of several microorganisms such as *Salmonella* Typhimurium, *E. coli, Staphylococcus aureus, Campylobacter jejuni,* oral pathogens and *Streptococcus mutans* biofilm formation [12,13]. The antimicrobial effect of GA on bacterial cells has been attributed to the effect of pH, chelation of divalent cations, and possible impairment of cell membrane permeability leading to loss of calcium ions and cell death in sensitive strains [13].

In the aspect of brucellosis treatment, several antibiotics are used for human brucellosis, however no effective drugs or natural medicines have been validated to treat animal brucellosis. Furthermore, current treatment for brucellosis remains complex and given the importance of the disease in livestock industries and public health burden, there is indeed an urgent need for identification of new antibacterial compounds that are natural, safe and effective against brucellosis.

In our previous studies, we successfully validated the potential use of phytochemical compounds for the treatment of animal brucellosis [14,15]. Here we attempt to investigate the effects of another phytochemical compound, GA on *B. abortus* infection in macrophages and mice for the purpose of finding an alternative safe treatment to control and prevent brucellosis.

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2. Materials and methods

2.1. GA preparation

GA (Sigma Chemical Co. USA) was dissolved in absolute ethanol (50 mg/ml), filtered through 0.45 μm membranes (Minisart, Sartorius Stedim Biotech, Germany) and diluted using sterile phosphate-buffered saline solution (PBS, pH 7.4).

2.2. Bacterial strain

The standard wild-type *B. abortus* 544 (ATCC 23448), a smooth, virulent *B. abortus* biovar 1 strains was used and cultivation was carried out in Brucella broth (Becton Dickinson, USA) or on Brucella agar grown at 37 °C with vigorous shaking until stationary phase. The viable counting of bacteria was assessed by plating serial dilutions on agar plates.

2.3. Cell culture

RAW 264.7 cells (ATCC, Rockville, USA) were grown at 37 °C with 5% CO_2 atmosphere in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 μmol/ml L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all provided by Gibco, Invitrogen, USA). The cells were seeded in tissue culture plates at a concentration of 1×10^5 cells per well and incubated for 24 h. Culture medium was changed into fresh medium without antibiotics before conducting all infection assays.

2.4. Cytotoxicity assay

RAW 264.7 cells were cultured in a 96-well plate in the presence of different concentrations of GA (0, 50, 100, 200 and 400 μg/ml) for 48 h. Cell viability was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, USA) assay.

2.5. F-actin staining

RAW 264.7 cells were cultured in a 12-well plate with 18-mm diameter glass coverslips (Fisher Scientific, USA) and pretreated with GA (100 μg/ml) for 2 h. To observe F-actin reorganization, same protocol was performed as previously described [16,17] with slight modifications. Briefly, bacteria grown at stationary phase (1×10^9) were suspended into 1 ml fluorescein isothiocyanate (FITC, 0.5 mg/ml) (Sigma-Aldrich, USA) in 50 mM sodium carbonate-100 mM sodium chloride at pH 9.0, and incubated at 37 °C for 20 min. The cells were infected with FITC-conjugated *B. abortus* for 10 min, fixed with 4% paraformaldehyde, incubated at 37 °C for 1 h, and permeabilized with 0.1% Triton X-100 for 10 min at 22 °C. The samples were incubated with a blocking buffer (2% goat serum in PBS) for 30 min and stained with 0.1 μM rhodamine-phalloidin (Cytoskeleton, Inc. USA) for 30 min at 22 °C. Fluorescence images were collected using a laser scanning confocal microscope (Olympus FV1000, Japan) and processed using FV10-ASW Viewer 3.1 software. The negative and positive control cells were treated with PBS without and with *B. abortus* infection, respectively.

2.6. FACS assay

RAW 264.7 cells were cultured and pretreated with GA (100 μg/ml) in six-well plates. The procedure for FACS assay was performed as previously described [5]. The F-actin content was quantified as log-scaled fluorescence histograms from 10,000 cells by FACS analysis using FACSCalibur flow cytometer (BD Biosciences, USA) and the average F-actin content was expressed as the mean of the fluorescence intensity. Preparation of negative and positive control cells was similar as that of F-actin staining.

2.7. Western blotting

Western blotting methods were performed as previously described [18]. The RAW 264.7 cells were lysed using radioimmunoprecipitation assay (RIPA) buffer with 1% protease inhibitor cocktail overnight and protein concentration was measured using Bradford protein assay (Bio-Rad, USA). Denatured proteins (10 μg) were separated by SDS-PAGE on a 10% polyacrylamide gel and then transferred onto Immobilon-P membranes (Millipore, USA). The membrane was blocked with 5% blocking buffer in 1× Tris-buffered saline-Tween 20 (TBS-T) (20 mM Tris-HCl, 150 mM NaCl, Tween 0.1%, pH 7.6) for 30 min at room temperature and incubated with phospho-specific antibodies against ERK1/2 (Thr183/Tyr185), JNK (Thr183/Tyr185) and p38α (Thr180/Thr182) overnight at 4 °C. Pan antibodies and β-actin antibody were applied to verify equivalent amounts of protein loaded per lane (all antibodies were provided by Cell Signaling, USA). The membrane was incubated with horseradish peroxidase (HRP)-conjugated protein G (Thermo Scientific, USA; 1:1000 dilution) in 5% blocking buffer for 2 h, washed with 1× TBS-T and detected using a luminal-coumaric acid-H_2O_2 detection solution (Atto Corporation, Japan). Image analysis was performed using NIH ImageJ software. Preparation of negative and positive control cells was similar as that of F-actin staining.

2.8. Protection experiment and immune response analysis in mice

To evaluate protective effects, four groups of five mice each, six to eight-week-old pathogen-free female ICR mice kept at 23 ± 1 °C with a 12 h light/dark cycle and acclimatized for one week prior to experiment were used in this study. Two groups of mice were not infected and were given orally with PBS (negative control) or GA (100 μg/ml) while the other two groups of mice were infected intraperitoneally with *B. abortus* (2×10^4 CFUs) and were given orally with PBS (positive control) or GA (100 μg/ml). Oral treatment was given using a gavage needle and was done three days prior to infection until 14 days post-infection. The mice were sacrificed, the blood was collected from the heart and the spleens were removed and weighed. The spleens of infected mice were homogenized, serially diluted in PBS and plated on Brucella agar to count the number of CFU in each infected group. The unit of protection (U) was obtained by subtracting the mean $\log_{10}$ CFU of the experimental group from the mean $\log_{10}$ CFU of the untreated positive control group. All the procedures described were reviewed and approved by the Animal Ethical Committee of Gyeongsang National University (Authorization Number GNU-120423-M0012).

To evaluate immune response, the level of IL-12p70, TNF, IFN-γ, MCP-1, IL-10 and IL-6 were analyzed from each serum sample using mouse inflammation cytometric bead array (CBA) kit (BD Biosciences, USA) according to manufacturer's instructions. The cytokines were measured on a FACSCalibur flow cytometer (BD Biosciences, USA) in combination with a representative data acquisition and analysis package (BD CellQuest™ software). These cytokines were selected based on their influence in the process and the outcome of *Brucella* infection [19].

2.9. Statistical analysis

The data are expressed as the mean ± standard deviation (SD) for the replicate experiments. Student's *t*-test was used to make a statistical comparison between the groups. Results with $P < 0.05$ were considered statistically significant.