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Nocodazole treatment interrupted *Brucella abortus* invasion in RAW 264.7 cells, and successfully attenuated splenic proliferation with enhanced inflammatory response in mice

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ABSTRACT

Brucellosis is one of the most important and widespread zoonosis worldwide responsible for serious economic losses and considerable public health burden. In this study, we investigated the modulatory effect of a microtubule-inhibitor, nocodazole, on *B. abortus* infection in murine macrophages and in a mouse model. Nocodazole activated macrophages and directly inhibited the growth of *Brucella* in a dose-dependent manner. Nocodazole increased adhesion but reduced invasion and intracellular growth of *Brucella* in macrophages although it did not affect co-localization of *Brucella* with LAMP-1. In addition, nocodazole negatively affected actin polymerization, and weakly activated ERK and p38α but significantly activated JNK in non-infected cells. After subsequent infection, nocodazole weakly inhibited activation of ERK and p38α. For the *in vivo* tests, nocodazole -treated mice displayed elevated levels of IFN-γ, MCP-1 and IL-10 while *Brucella*-infected nocodazole -treated mice showed high levels of TNF, IFN-γ, MCP-1, IL-10 and IL-6 as compared to controls. Furthermore, nocodazole treatment reduced inflammation and *Brucella* proliferation in the spleens of mice. These findings highlight the potential use of nocodazole for the control of brucellosis although further investigations are encouraged to validate its therapeutic use in animal hosts.

1. Introduction

Brucellosis, caused by *Brucella* species, is a highly contagious zoonosis which affects livestock, wild animals and humans [1]. The disease is caused by *Brucella* which mainly affects the reproductive tract of its natural hosts and exerts a significant socioeconomic impact due to decreased animal productivity [2]. In humans, it causes acute inflammation and diverse pathological manifestations such as undulating fever, sweats, headaches, back pains and physical weakness leading to endocarditis, arthritis, meningitis, osteoarticular complications and neurological disorders, with approximately half a million new cases occurring each year worldwide [3,4].

The virulence of *Brucella* is dependent on its capacity to attach and eventually invade different host cells [5]. Adhesion is one of the initial and crucial stages of the infectious process and the binding of *Brucella* to host cells promotes reorganization of the actin cytoskeleton to enhance invasion and entry requires full activation of a mitogen-activated protein kinase (MAPK) signaling pathway [6]. Once inside the host cells, *Brucella* is exposed to harsh diverse environment, hence it develops multiple strategies to evade host immune response mechanisms and establish persistent infection and replication within host [7]. The pathogen can control the intracellular trafficking of their vacuoles, *Brucella*-containing vacuoles (BCVs), and modify its outer membrane by installing phosphatidylcholine to avoid fusion with lysosome, and can synthesize cyclic β-1,2-glucans that interact with the lipid rafts to prevent fusion between phagosome and lysosome for successful survival and intracellular replication [4,7].

Abbreviations: BCVs, *Brucella*-containing vacuoles; MAPKs, mitogen-activated protein kinases.

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Several important roles of microtubules have been identified: participation in many cell responses such as cell division, migration and intracellular signal transduction; stabilization and destabilization of these components directly affects various cellular signaling processes; and involvement in a number of signal transduction pathways such as NF-κB, JNK (c-Jun N-terminal kinase) and MAPK [8]. The cellular localization of microtubules and actin filaments are known to depend on one another and were found to be highly dependent on each other of which the removal of either component would dramatically change the organization of the other [9]. Furthermore, increasing observations of microtubule-dependent invasion systems have been reported in several microorganisms including *Campylobacter jejuni, Citrobacter freundii, Klebsiella pneumonia, Orientia tsutsugamushi, Haemophilus influenzae* and *Actinobacillus actinomycetemcomitans* [10]. Consequently, we investigated the effect of a microtubule-inhibitor nocodazole in the invasion and intracellular trafficking of *B. abortus* in murine macrophages, and subsequently in the immune response using a mouse model.

2. Materials and methods

2.1. Nocodazole preparation

Nocodazole was purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA), dissolved in dimethylsulfoxide (DMSO) (10 mg/ml) (Amresco, LLC, OH, USA) and further diluted using sterile phosphate-buffered saline solution (PBS, pH 7.4).

2.2. Bacterial strains and cell culture

B. abortus 544 (ATCC 23448) obtained from the Laboratory of Bacteriology Division in Animal and Plant Quarantine Agency, Korea was cultivated in *Brucella* broth (Becton Dickinson, Sparks, MD, USA) at 37 °C with vigorous shaking until stationary phase. Routine cultivation was carried out in Brucella broth (Becton, Dickinson and Company, MD, USA) or agar (1.5%). The RAW 264.7 cells (ATCC, Rockville, MD, USA) were grown at 37 °C with 5% CO_2 atmosphere in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Invitrogen, Grand Island, NY, USA). The cells were seeded in tissue culture plates at a concentration of 1×10^5 per well and prior to bacterial infection, culture medium was changed without antibiotics.

2.3. Cytotoxicity assay

The cells were pre-treated with different concentrations of nocodazole (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 5, 10 μg/ml) in a 96-well cell culture plate for 48 h and analysis of cytotoxicity was performed using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Amresco, OH, USA) by incubation with 0.5 mg/ml MTT as previously described [11]. Fresh culture medium with 0.1% DMSO in PBS was used as a control in all of the succeeding experiments.

2.4. Morphologic changes in macrophages

RAW 264.7 cells were cultured in 12-well plates with 18-mm diameter glass coverslips (Marienfeld, Lauda-Konigshofen, Germany) overnight and were pre-treated with the highest non-cytotoxic concentration of nocodazole (0.4 μg/ml) for 0, 2, 4 and 8 h. One hundred cells were selected randomly and the morphologically changed cells were counted.

2.5. Bactericidal analysis

Bacteria (2×10^4 colony forming units, CFU/ml) were added to different concentrations of nocodazole (0, 0.2, 0.4, 0.8 and 1 μg/ml) and incubated at 37 °C for 0, 2, 4 and 24 h. The samples were serially diluted on Brucella agar and incubated at 37 °C for 3 days. Bacterial survival rates were expressed as a percentage of the survival rate of the treated sample relative to untreated control.

2.6. Internalization assay

RAW 264.7 cells were pre-treated with non-cytotoxic concentration of nocodazole (0.4 μg/ml) for 4 h prior to infection. Cells were then infected with *B. abortus* at a MOI of 100, centrifuged at 150×*g* for 10 min and incubated at 37 °C with 5% CO_2 in air. After 0 and 30 min, macrophages werSe washed once with PBS and incubated with medium containing 10% (v/v) FBS and gentamicin (30 μg/ml) (Gibco, Invitrogen Corp., NY, USA) for 30 min. Cells were then washed and lysed with distilled water. Serial dilutions of cell lysates were spread onto Brucella plates to determine CFUs.

2.7. Intracellular growth assay

Bacteria were deposited onto macrophages as described for internalization assay and incubated for 1 h. Cells were then washed and incubated for 2, 24 or 48 h with medium containing 10% (v/v) FBS, gentamicin (30 μg/ml) and in the presence of nocodazole (0.4 μg/ml). Washing, lysis, plating procedures were the same as for internalization assay.

2.8. Adherence assay

To inhibit bacterial internalization, pre-treated cells in 12-well plate with 18-mm diameter glass coverslips were incubated with cytochalasin D (500 μg/ml) (Sigma-Aldrich, MO, USA) for 40 min as previously described [12]. Cells were washed and bacteria were deposited as described for internalization assay. After 30 min, cells were washed, fixed with 4% paraformaldehyde (iNtRON Biotechnology, Inc., Gyeonggi-do, Korea) at 37 °C for 30 min and permeabilized with methanol (Fisher Scientific Korea Ltd., Seoul, Korea) at −20 °C for 10 s. Cells were incubated with anti-*B. abortus* polyclonal rabbit serum (1:500) and then with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) (1:500, Sigma-Aldrich, St. Louis, MO, USA). Incubation with antibodies was performed for 1 h at 37 °C. Cells were placed in fluorescent mounting medium (DakoCytomation, Dako North America, Inc., Carpinteria, CA, USA).

2.9. F-actin staining

Pre-treated cells were prepared as described for adherence assay and infected with FITC-conjugated *B. abortus* for 10 min, fixed and permeabilized with 0.1% Triton X-100 for 10 min at 22 °C. Macrophages were incubated in blocking buffer (2% goat serum in PBS) at room temperature for 30 min and stained with 0.1 μM rhodamine-phalloidin (Cytoskeleton, Inc., Denver, CO, USA) for 30 min at 22 °C. Cells were washed, mounted and visualized by fluorescence microscopy.

2.10. F-actin analysis

Pre-treated macrophages were prepared as described for adherence assay in 6-well plate. Bacteria were deposited onto cells as described for F-actin staining, fixed with 4% paraformaldehyde at 37 °C for 30 min, and then permeabilized and stained with 20 μg/ml