Biomedicine & Pharmacotherapy 101 (2018) 100–106

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Biological effects of kojic acid on human monocytes *in vitro*

Josineide P. Da Costa[a,b], Ana Paula D. Rodrigues[c], Luis Henrique S. Farias[c], Paula Cristina R. Frade[a], Bruno José Martins Da Silva[a,b], Jose Luiz M. Do Nascimento[d,e,f], Edilene O. Silva[a,b,*]

[a] Federal University of Pará, Institute of Biological Sciences, Laboratory of Structural Biology, Belém, Pará, Brazil
[b] National Institute of Science and Technology in Structural Biology and Bioimaging, Rio de Janeiro, Rio de Janeiro, Brazil
[c] Laboratory of Electron Microscopy, Evandro Chagas Institute, Department of Health Surveillance, Ministry of Health, Belém, Pará, Brazil
[d] Federal University of Pará, Institute of Biological Sciences, Laboratory of Neurochemistry Molecular and Cellular, Belém, Pará, Brazil
[e] Neuroscience Research Group, University CEUMA, São Luís, Brazil
[f] National Institute of Science and Technology in Neuroimmunomodulation (INCT – NIM), Rio de Janeiro, Rio de Janeiro, Brazil

ARTICLE INFO

Keywords:
Human monocyte
Morphological alterations
Autophagy
Natural product

ABSTRACT

Monocytes are mononuclear phagocytes in peripheral blood that can differentiate into macrophages and dendritic cells. Macrophages play a specific role in the inflammatory process and are essential for the innate response. Given the important role of monocytes/macrophages in the immune response, this study aimed to evaluate the activity of kojic acid (KA), a natural product of certain fungal species, on human peripheral blood monocytes *in vitro*. Purified monocytes isolated from human blood were incubated with KA (50 μg/mL for 48 h) and analyzed by light microscopy, scanning electron microscopy, transmission electron microscopy and flow cytometry. Host cell cytotoxicity was measured by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. KA treatment induced morphological alterations in monocytes, such as increased cell size, as well as numerous cellular projections. Furthermore, flow cytometry revealed increased labeling of cell surface EMR1-F4/80 but decreased labeling of CD11b and CD14. KA also promoted increased IL-6 cytokine production but did not cause cytotoxic effects in monocytes. In conclusion, our results show that KA promotes the differentiation of monocytes into macrophages and can act as an immunomodulatory agent.

1. Introduction

Monocytes, mononuclear phagocytes that are present in peripheral blood, can differentiate into macrophages [1] as well as dendritic cells [2–4]. The differentiation process begins when a monocyte migrates from circulating blood into surrounding tissues [5]. A number of cytokines that aid in the differentiation process are released during migration, including macrophage colony-stimulating factor (M-CSF), tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) [3,6]. IL-6 secreted by monocytes and macrophages also contributes to the removal of infectious agents and restoration of damaged tissues by activating the immune response [7].

During the differentiation/maturation process, monocytes undergo several morphological changes. Blood monocytes display few filopodia, a reduced cytoplasmic area and few organelles [8–11]. However, cells that are differentiating/maturing exhibit an increased size, numerous cytoplasmic projections, enhanced spreading ability, autophagy induction (increased LC3b protein) and surface protein pattern changes (increased EMR1-F4/80 and CD80 and reduced CD11b and CD14) [12–15].

Macrophages are differentiated cells that are important for innate immunity, secretion of cytokines, elimination of several intracellular pathogens after activation and establishment of an effective immune response [4,16]. Moreover, studies have show that by providing protection against intracellular pathogens, such as fungi, bacteria and protozoans, monocytes (non-differentiated circulating cells) are also important regulators of homeostasis [17]. It is interesting to note that modulation of monocyte differentiation into macrophages can be induced by M-CSF or other biologically active compounds, including natural products [18–21].

Kojic acid (KA), a natural product produced by *Aspergillus*, *Penicillium* and *Acetobacter* fungi [22], has various biological properties, such as potent tyrosine-inhibition properties as well as antioxidant and antitumor activities [23–26]. Previously, we reported that KA can

* Corresponding author at: Federal University of Pará, Institute of Biological Sciences, Laboratory of Structural Biology, Augusto Corrêa Av., 01, Guamá, 660975-110, Belém, Pará, Brazil.
E-mail address: edilene@ufpa.br (E.O. Silva).

https://doi.org/10.1016/j.biopha.2018.02.036
Received 27 November 2017; Received in revised form 29 January 2018; Accepted 9 February 2018

induce activation of murine peritoneal macrophages by increasing reactive oxygen species (ROS) production without causing cytotoxic effects [27]. Additionally, KA exerts antileishmanial activity against *Leishmania (L.) amazonensis* both *in vitro* and *in vivo* [28]. It is probable that KA's capacity to promote death in *Leishmania* and to induce increased ROS production is due to immunomodulation and to the induction of proinflammatory cytokines.

In the present study, we investigated the modulatory action of KA on human monocytes to determine if this metabolite can induce morphological changes and exhibits immunomodulatory activity toward these cells.

2. Materials and methods

2.1. Kojic acid (KA)

KA (SIGMA®) was diluted to 1 mg/mL in Dulbecco's Modified Eagle Medium (DMEM). This was used as the standard solution to obtain the concentration (50 µg/mL) chosen for this study, as based on our current results and previous data from our group [27].

2.2. Isolation of human mononuclear cells

Monocytes were isolated from peripheral blood (collected in EDTA) that was obtained from healthy humans (Blood collection center, Belém, Para, Brazil). The cells were separated using Histopaque®-1077 (SIGMA®) according to Smit et al., [20]. The cells were incubated at 37 °C under 5% CO_2 in a humidified atmosphere with Dulbecco's Modified Eagle's Medium (DMEM) (pH 7.2).

Subsequently, non-adherent cells were removed, and 50 µg/mL KA was added for 48 h. Positive control cells were treated with 100 nM M-CSF (SIGMA®) and maintained in parallel. The experiments and study were performed in accordance with a current Brazilian resolution related to research involving human subjects (Resolution number 466/2012/ Ministry of Health, Brazil). The protocol was approved by the Committee for Ethics in Research (CEP) through the platform Brazil and Institute of Health Sciences of the Federal University of Pará (CEP/ICS/UFPA- Number CAEE 42632915.0.0000.0018).

2.3. Measurement of cell viability using the MTT assay

The MTT assay is based on the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (SIGMA®) to formazan. The procedure was performed following a protocol described by Fotakis and Timbrell. [29] with some modifications. Briefly, monocytes were treated with 10–100 µg/mL KA for 48 h. Subsequently, the cells were incubated with 0.5 mg/mL MTT for 3 h at 37 °C in a humidified atmosphere containing 5% CO_2. Subsequently the incubation was added dimethyl sulfoxide (DMSO) and gentle shaking for 5 min to achieve complete dissolution. The resulting solutions were transferred to 96-well plates, and the absorbance was recorded at 570 nm using a microplate spectrophotometer (BIORAD Model 450 Microplate Reader).

2.4. Light microscopy (LM)

Monocytes were cultured and treated before being divided into three groups: control (nontreated cells), cells treated with KA (50 µg/mL for 48 h), and cells treated with M-CSF (100 nM). Cells were fixed in a solution containing 3% paraformaldehyde in PHEM buffer (5 mM magnesium chloride, 70 mM potassium chloride, 10 mM EGTA, 20 mM HEPES, 60 mM PIPES) at 0.1 M and pH 7.2. They were then stained with Giemsa (SIGMA®) for 30 min at room temperature before being covered with Entellan® (Merck). Cells were analyzed using an Olympus BX41 microscope.

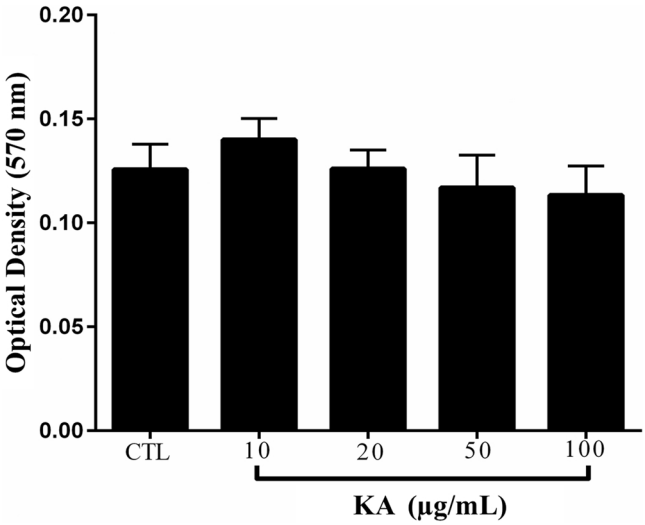

Fig. 1. Viability of macrophages treated with kojic acid measured by the MTT assay after 48 h of treatment. KA does not induce cytotoxic effects in monocytes. Viability was obtained for various concentrations of KA (10–100 µg/mL). The viability of treated and untreated cells is reported as absorbance, which was recorded as optical density (OD) at 570 nm. Data were analyzed by ANOVA followed by the Tukey test ($p < 0.05$).

2.5. Scanning electron microscopy (SEM)

Human monocytes were cultured on coated coverslips and treated with 50 µg/mL KA for 48 h. The samples were processed for SEM, as described Rodrigues et al., [27].

2.6. Transmission electron microscopy (TEM)

Control and treated human monocytes (50 µg/mL KA for 48 h) were cultured in flasks, washed with phosphate-buffered saline (PBS) and processed for conventional TEM, as described by Da Silva et al. [30]. Thin sections were obtained and stained with uranyl acetate and lead citrate. The sections were analyzed using a Zeiss LEO 906E TEM.

2.7. Morphometric analysis

Morphometric analysis was performed using ImageJ (NIH) software, and pictures were obtained by scanning electron microscopy (SEM). The cytoplasmic areas of the control and treated cells (50 µg/mL KA for 48 h) were analyzed as previously described by Sokol et al. [31] and Papadopulos et al. [32].

2.8. Detection of surface markers EMR1-F4/80, CD14, CD11b and CD11c by flow cytometry

Human monocytes (1×10^6) were cultured and fixed for 30 min in 3% freshly prepared formaldehyde in PHEM buffer. The control and treated cells (50 µg/mL) were incubated with 50 mM NH_4Cl for 1 h and with glycine buffer (0.1 M) for 10 min and then washed with PBS (pH 8.0) containing 1% bovine serum albumin (BSA) and 0.01% Tween 20 (PBS-BSA-Tw). Subsequently, the cells were incubated for 1 h with a rat anti-mouse/human EMR1-F4/80 monoclonal antibody (Santa Cruz Biotechnology®) or an antibody against CD11b (BD Bioscience®) or CD11c (BD Bioscience®) diluted 1:50 in PBS-BSA-Tw. The cells were washed with PBS-BSA-Tw and incubated for 45 min with PE-goat anti-rat IgG (Abcam®) diluted 1:50 in PBS-BSA-Tw and analyzed by flow cytometry. Data were acquired with a FACSCanto II cytometer using Diva software from Becton Dickinson (San Jose). The results were analyzed by WinMDI, version 2.9 (Joseph Trotter). Positive control cells treated with M-CSF (100 nM) were maintained in parallel. All experiments with treated and untreated cells were performed at least three