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Pharmacological effects of mitraphylline from *Uncaria tomentosa* in primary human monocytes: Skew toward M2 macrophages

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ABSTRACT

Ethnopharmacological relevance: *Uncaria tomentosa* (Willdenow ex Roemer & Schultes) DC. (Rubiaceae) is a Peruvian thorny liana, commonly known as "cat's claw", and traditionally used in folk medicine to deal with several inflammatory diseases. Mitraphylline (MTP) is the most abundant pentacyclic oxindolic alkaloid (POA) from *U. Tomentosa* and has been reported to modify the inflammatory response. Herein, we have sought to identify the mechanisms underlying this modulatory effect of MTP on primary human monocytes and its ability to regulate differentiation processes on human primary monocyte and monocyte-derived macrophages.

Material and methods: In vitro studies with human primary monocytes and monocyte-derived macrophages were performed. Monocytes and M0 macrophages were exposed to MTP (25 μM) and LPS (100 ng/mL). M0 macrophages were polarized to M1 and M2 phenotypes in the absence or presence of MTP. The activation state of monocytes/macrophages was assessed by flow cytometry, gene expression and protein analysis of different specific markers.

Results: In human primary monocytes, the incubation of MTP for 24 h reduced the number of classical ($CD14^{++}CD16^{-}$) and intermediate ($CD14^{++}CD16^{+}$) subsets when compared to untreated or LPS-treated cells. MTP also reduced the chemotactic capacity of human primary monocytes. In addition, MTP promoted the polarization of M0 macrophages toward an anti-inflammatory M2 phenotype, the abrogation of the release of pro-inflammatory cytokines such as TNFα, IL-6 or IL-1β, as well as the restoration of markers for M2 macrophages in LPS-treated M1 macrophages.

Conclusions: Our results suggest that MTP may be a key modulator for regulating the plasticity of monocytes/macrophages and the attenuation of the inflammatory response.

1. Introduction

Uncaria tomentosa (Willdenow ex Roemer and Schultes) DC and its relative *Uncaria guanensis* (Aublet) Gmell. (Rubiaceae) are plants widely used in folk and complementary medicine under the names of "Cat's claw". Uncarias are vines naturally growing in Peru and some other countries of South and Central America, and have their numerous applications in both human and animal medicine. *U. tomentosa* has been previously reported to deal with several inflammatory diseases (Müller and Kanfer, 2011; Rosenbaum et al., 2010), modulating the immune system, and antitumor activities (García-Gimenez et al., 2010; Rojas-Duran et al., 2012; Dietrich et al., 2014). Owing to the systematically growing applications of *U. tomentosa* in therapy, it is important to better recognize its pharmacological properties and safety of using.

Cat's claw contains more than 50 chemical constituents, including oxindole and indole alkaloids, polyphenols, among others (Heitzman et al., 2005). The majority alkaloids of *Uncaria* are indole and oxindole families (Laus, 2004), which are well recognized as phytochemical markers of this species with relevant pharmacological activities (Heitzman et al., 2005). Mitraphylline (MTP) is an oxindole alkaloid and the most ubiquitous alkaloid being present in 20 of 34 *Uncaria* species (Heitzman et al., 2005). Most of pharmacological studies have been focused on the fractions of either a plant species or a "crude drug", considered as a

Abbreviations: MTP, mitraphylline; POA, pentacyclic oxindolic alkaloid; *U. tomentosa*, *Uncaria tomentosa*; LPS, lipopolysaccharide; TNFα, tumor necrosis factor alpha; IL, interleukin; IFNγ, interferon gamma; CCR2, chemokine (C–C motif) receptor 2; CCL2, chemokine (C–C motif) ligand 2; MCSF, macrophage colony-stimulating factor

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preparation from either a single or a mixture of *Uncaria* plants. However, fewer studies, if any, have paid attention to the bioactivity of isolated compound(s) such as MTP. Our research team have previously reported the isolation of MTP from the dried inner bark of U. *tomentosa*, the elucidation of its structure by NMR spectroscopy analysis, and its antitumor properties on human sarcoma and breast cancer cells (García-Prado et al., 2007). However, little is known on the potential of MTP to mitigate the inflammatory response.

Monocytes are particularly involved in inflammatory processes (Shi et al., 2011). They are classified into three subsets: classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$, and non-classical $CD14^{+}CD16^{++}$ monocytes (Ziegler-Heitbrock et al., 2010). So far, classical monocytes represent the major fraction, about 85% of total monocytes that highly express the migratory chemokine receptor CCR2 (Ingersoll et al., 2010; Wong et al., 2012). They are professional phagocytes giving rise to classical M1 macrophages, which generate reactive oxygen species and secrete cytokines (TNFα, IL-1β, IL-6) in response to LPS during infection or inflammation (Mossig et al., 2009; Wong et al., 2012; Zwada et al., 2011). Intermediate monocytes display highest levels of CCR5, TLR4, CD163, and HLA-DR during activation and also secrete pro-inflammatory cytokines (Wong et al., 2012; Shantsila et al., 2011). Non-classical monocytes are less granular and smaller in size, with lower expression of CCR2 than classical or intermediate subsets (Cros et al., 2010; Wong et al., 2012; Shantsila et al., 2011). These monocytes rich in CD16 are functionally involved in tissue repairing, patrolling, wound healing, and have the tendency to be polarized into non-classically M2 macrophages with an anti-inflammatory phenotype in response to a variety of stimuli, including IL-4 (Benoit et al., 2008; Gordon and Martinez, 2010).

In the present study, we further investigated whether MTP may act as an immune-modulator and anti-inflammatory effector on human primary monocytes and monocyte-derived macrophages.

2. Material and methods

2.1. Bark extract

The plant material was collected in the Peruvian forest and was provided by Dr Carlos S González and was identified in the Botanic Department of the San Lorenzo Chemical Science School from the Asuncion University in Paraguay. The plant extract was obtained and the compound MTP identified as previously described (García-Prado et al., 2007). Briefly, 500 g of *U. tomentosa* dried inner bark was treated with ammonium hydroxide and extracted with 500 mL of dichloromethane for 3 times. After filtration, the obtained solution was concentrated in vacuo to afford a residue, which was dissolved in a hydrochloric acid solution (3%). Ammonium hydroxide and dichloromethane were added again. After concentration in vacuo, the purified alkaloid fraction was obtained as a brown residue and the yield was 0.1%.

2.2. Isolation and identification of MTP

The dried residue of alkaloid fraction (0.5 g) was subjected to a silica gel column chromatography, compacted with silica gel 0.063–0.2 mm (0.8 × 25 cm), followed by a gradient elution with various mixtures of n-hexane, dichloromethane, and methanol. Twenty-five fractions of about 15 mL each were collected. 200 mg of white crystal (MTP) was obtained in the fractions 14–16 (eluted with dichloromethane/methanol) that corresponded to 87.2% of the total alkaloids. EIMS and ^{1}H and ^{13}C NMR experiments were carried out for its identification using an AVANCE 500 spectrophotometer. The following 2D NMR experiments COSY-DQF, (^{1}H–^{13}C)–HSQC, (^{1}H–^{13}C)–HMBC, and NOESY correlation were used to elucidate its structure. Furthermore, the 15N chemical shift of the isomeric oxindole alkaloids, (^{1}H–15N)–HMBC, was necessary to facilitate its characterization. The solvent used for NMR spectra was CDCl3 (García-Prado et al., 2007).

2.3. Blood collection and monocyte isolation

This study was conducted according to the guidelines of good clinical practice. Peripheral venous blood was isolated from healthy adult volunteers (< 35 years old) from the Hospital Virgen del Rocio at Seville. The investigation conformed to the principles outlined in the Helsinki Declaration of the World Medical Association. Donors declared that they were non-smokers and were not taking any medication. Blood samples were immediately collected into K_3EDTA-containing Vacutainer tubes (Becton Dickinson, NJ, USA) and peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over Ficoll Histopaque gradient (Sigma-Aldrich Chem, MO, USA). Monocytes were then isolated from PBMCs using positive selection with CD14 MicroBeads according to the manufacturer's instructions (MACS, Myltenyi Biotec, Madrid, Spain). Monocytes were tested for purity by CD14 fluorescein isothiocyanate labeling and fluorescence-activated cell sorter (FACS) analysis using a FACScanto II flow cytometer and FACSDiva software (Becton Dickinson Immunocytometry Systems, CA, USA) (Valera et al., 2011). Following isolation, the cells were suspended in a RPMI 1640 medium supplemented with L-glutamine, penicillin, streptomycin, and 1% heat-inactivated FCS. The monocytes were used within 24 h after isolation for the experiments.

2.4. Monocyte differentiation and polarization into M1 and M2 macrophages

Monocytes were induced to differentiate for 6 days in the presence of recombinant human M-CSF (25 ng/mL) to obtain M0 macrophages. These cells were then cultured in RPMI 1640 supplemented with L-glutamine, penicillin, streptomycin, and 10% heat-inactivated FCS. For M1 and M2 polarization, M0 macrophages were exposed to LPS (100 ng/mL) plus IFNγ (20 ng/mL) and to IL-4 (20 ng/mL), respectively, for additional 24 h. MTP (25 μM) was also added to the medium as indicated.

2.5. Cell viability

Monocytes and macrophages seeded in 96-well plates (1×10^5 cells/well) were incubated in the presence (or absence) of different MTP concentrations for 24 h. At the end of the exposure time, cell viability was analyzed by Annexin V binding and using a FACScanto II flow cytometer with a CellQuest software (BD).

2.6. Nitrite and cytokine production

Cells in 24-well plates were treated (or untreated) with MTP (25 μM), and 30 min later stimulated with LPS (100 ng/mL) for 24 h. The culture supernatants (100 μL) were transferred to a 96-well assay plate mixed with Griess reagent (Sigma-Aldrich Chem) and incubated for 15 min at room temperature. The amount of nitrite, as an index of NO generation (Csonka et al., 2015), was determined by the absorbance at 540 nm in an ELISA reader (BioTek, Bad Friedrichshall, Germany). After the extrapolation from a standard curve with sodium nitrite, the results were expressed as the percentage of nitrite compared with that of cells treated with only LPS. Dexamethasone (1 μM, Sigma-Aldrich Chem) was used as positive control.

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