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Cucurbitacin D is a new inflammasome activator in macrophages

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ABSTRACT

We previously reported that cucurbitacin D isolated from *Trichosanthes kirilowii* has anti-tumor roles to leukemia cells. However, the effect of cucurbitacin D on immune cells is not fully understood although there is no toxic activity to normal cells. In this study, immunomodulating activities of cucurbitacin D were investigated in macrophages. Cucurbitacin D could increase LPS-induced interleukin (IL)-1β production in culture supernatant of THP-1 cells, peritoneal exudate cells (PECs), bone marrow derived macrophages (BMDMs), and RAW264 cells. At the transcriptional level, cucurbitacin D enhanced LPS-induced IL-1β mRNA expression through activation of ERK1/2 mitogen-activated protein kinases (MAPKs). At the posttranscriptional level, the activation of caspase-1 induced by cucurbitacin D has also been demonstrated following treatment with a caspase-1 inhibitor and siRNA. Importantly, cucurbitacin D has further been shown to induce inflammasome activation independent of ERK1/2 activation. Western blotting showed interaction of NOD-like receptor family, pyrin domain containing 3 (NALP3) and apoptosis-associated speck-like protein containing a caspase-activating and recruitment domain (ASC), suggesting activation of the inflammasome and a possible reason for activation of caspase-1. Taken together, these results suggest that cucurbitacin D could initiate immunomodulating activity in macrophages to lead to inflammasome activation as well as enhancement of LPS signaling.

1. Introduction

There has been growing interest in the use of traditional medicinal herbs (TMHs) as a potent source of new therapeutic drugs for the treatment of several diseases [1]. In Japan, standardized crude extracts and dried powder from TMHs were commercially supplied by some pharmaceutical companies; the combination of different TMH extracts has been clinically used for the therapeutic purposes [2]. Cucurbitacins, which derived from cucurbitaceae species, had been shown to inhibit tumor growth in vitro and in vivo [3]. We have reported that cucurbitacin D isolated from *Trichosanthes kirilowii* has anti-tumor activities to leukemia cells but not to peripheral blood lymphocytes (PBL) from healthy donors [4]. There is no toxic activity to PBL, however; it is still unclear whether cucurbitacin D affects normal cells or modulates immune activity.

IL-1β is a pro-inflammatory cytokine secreted by activated macrophages and monocytes and is involved in cell growth, tissue remodeling and regulation of immune responses [5]. Based on its immunoregulatory activity as a co-stimulator of T cell functions, IL-1β has been shown to induce the tumor growth inhibition of fibrosarcomas and melanomas [6,7]. IL-1β is also an important growth factor for B cell proliferation and antibody production [5].

The production of IL-1β is regulated at transcriptional and posttranscriptional levels. Toll-like receptor (TLR) ligands, such as LPS, initiate the synthesis of inactive IL-1β precursor (pro-IL-1β) at the transcriptional level through activation of MAPKs such as ERK1/2 [8,9]. At the posttranscriptional level, pro-IL-1β accumulated in the cytoplasm was cleaved by activated caspase-1 into active IL-1β (mature IL-1β), resulting in its release into the extracellular space [10]. Several studies demonstrated that assembly and activation of the inflammasome function to convert inactive pro-caspase-1 into active caspase-1 [11]. The molecular platform inflammasome for caspase-1 activation includes NLR family members and the ASC, which functions as the adaptor protein that links these NLRs through pyrin–pyrin domain, protein–protein interactions for the recruitment and activation of caspase-1 [12].

It is frequently observed that different dose of drugs have different effects. In this study, the induction of IL-1β from macrophages was investigated as a useful marker of immunomodulating activity, and we report that low dose of cucurbitacin D modulates immune responses of macrophages through enhanced activation of ERK1/2 and inflammasome.

Abbreviations: THMs, traditional medicinal herbs; PECs, peritoneal exudate cells; BMDMs, bone marrow derived macrophages; MAPKs, mitogen-activated protein kinases; NALP3, NOD-like receptor family, pyrin domain containing 3; ASC, apoptosis-associated speck-like protein containing a caspase-activating and recruitment domain; TLR, Toll-like receptor; LDH, lactate dehydrogenase.

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2. Materials and methods

2.1. Reagents and antibodies

Cucurbitacin D was purchased from Nacalai Tesque (Kyoto, Japan) and dissolved in 10% ethanol to make a stock concentration of 1 mg/ml. Endotoxin level was measured using an Endospecy ES-24S kit (Seikagaku biobusiness corporation, Tokyo, Japan) with commercial standards (CSE-L set, Seikagaku biobusiness corporation). Endotoxin levels in the cucurbitacin D solution were under the detection limit. LPS was from Sigma (St. Louis, MO). MAPK p38 inhibitor SB203580 (#559389), ERK kinase inhibitor U0126 (#662005), JNK inhibitor II (#420119) and caspase-1 inhibitor (#400010) were obtained from Calbiochem (San Diego, CA). Antibodies for p-p38 (#9211), p-ERK (#4370), p-JNK (#9251), p38 (#9212), ERK (#9108), JNK (#9252) and caspase-1 (#2225) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies for IL-1β (sc-7884), ASC (sc-22514), NALP3 (sc-66864) and siRNA for caspase-1 (sc-29922) and the control (sc-37007) were obtained from Santa Cruz (Santa Cruz, CA). Anti-β-actin antibody (A5441) was purchased from Sigma.

2.2. Mice

BALB/c mice (8–12 weeks) were purchased from Charles River Laboratories Japan (Yokohama, Japan). These mice were maintained in the Animal Research Center at the University of Occupational and Environmental Health, Japan. All animal experiments were performed according to the guidelines for the care and use of animals approved by the University of Occupational and Environmental Health, Japan.

2.3. Preparation of the cells and cell cultures

Peritoneal exudate macrophages (PECs) from BALB/c mice, which were harvested 3 days after the i.p. injection of 2 ml of 4% thioglycolate broth (Eiken Chemical Co., Tokyo, Japan) were cultured in RPMI 1640 medium (Nissui Seiyaku Co., Yokohama, Japan) supplemented with 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD), L-glutamine (2 mM) and penicillin (50 μg/ml)–streptomycin (50 U/ml) at 37 °C for 3 h. Nonadherent cells were detached by vigorous agitation and adherent cells were used as macrophages. The human monocytic THP-1 cell line and murine macrophage RAW264 cell line were obtained from Cell Resource Center of Tohoku University (Sendai, Japan) and RIKEN BioResource Center (Tsukuba, Japan), respectively. All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% FBS, L-glutamine (2 mM) and penicillin–streptomycin at 37 °C under 5% CO_2 and 95% air conditions. For preparation of BMDMs, bone marrow cells were obtained by flushing the femurs of mice. The prepared cells were cultured in RPMI 1640 medium supplemented with 20% FBS, L-glutamine, and penicillin–streptomycin at 37 °C under 5% CO_2 and 95% air conditions. Ten nanograms per milliliter of GM-CSF (PeproTech EC, London, UK) was added to the cultures on days 0 and 4. After 7 days in culture, nonadherent cells were vigorously washed out, and adherent cells were considered as macrophages. The purity of PECs and BMDMs was shown to be more than 95% as determined by F4/80- and Mac-1-positive flow cytometry.

2.4. ELISA

IL-1β production in the cell culture supernatant from macrophages was detected using ELISA kits (PeproTech EC for murine IL-1β; R&D Systems, Oxon, U.K. for human IL-1β).

2.5. Real-time quantitative PCR

THP-1 and BMDMs were stimulated with cucurbitacin D in the presence of LPS or not, and total RNA was extracted using the Aqua Pure RNA kit according to the manufacturer's instruction (Bio-Rad, Hercules, CA). First strand cDNA was synthesized from 5 μg of total RNA by Superscript II RNase H-reverse transcriptase (Life Technologies, Rockville, MD), using 0.25 μg of random primer (Life Technologies). This cDNA was used for quantitative PCR. Primers specific for IL-1β were used for analyzing gene expression using SYBR green method (Qiagen, Hombrechtikon, Switzerland). Gene expression was normalized to the expression of the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene. All primers were obtained from Qiagen and listed below: human IL-1β (QT00021385), human GAPDH (QT01192646), murine IL-1β (QT01048355) and murine GAPDH (QT01658692). A 10-μl aliquot of PCR products was electrophoresed on a 1.5% agarose gel. After ethidium bromide staining (Sigma), PCR products were visualized by UV illumination.

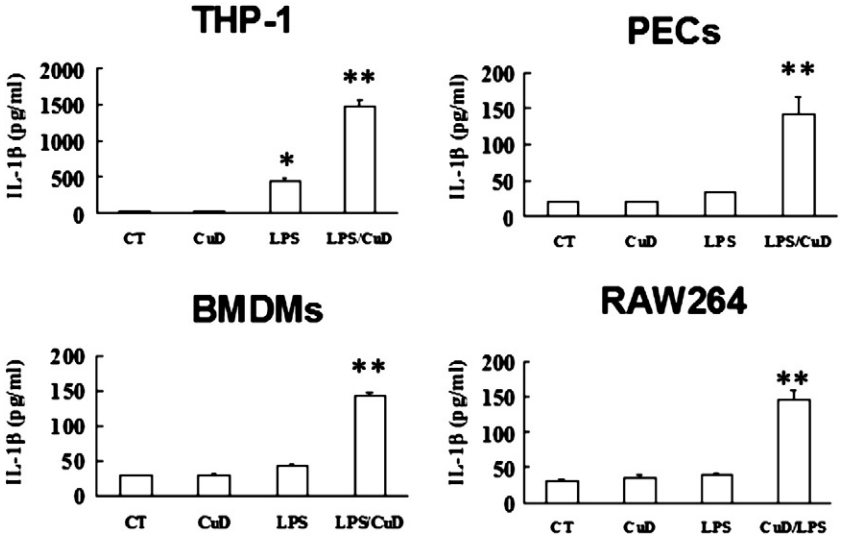

Fig. 1. Cucurbitacin D enhances LPS-induced IL-1β production in THP-1 cells, PECs, BMDMs and RAW264 cells. THP-1 (upper left), PECs (upper right), BMDMs (lower left) and RAW264 cells (lower right) (2.5×10^5) were stimulated with cucurbitacin D (CuD 0.25 μg/ml) in the presence or absence of LPS (1 μg/ml) for 6 h, and cell culture supernatants were assayed. The IL-1β production from macrophages was measured by ELISA. *Significantly increased compared to each control. **Significantly increased compared to LPS treatment. Results are representative of three similar experiments.