Toxicology Letters 245 (2016) 24–30

Contents lists available at ScienceDirect

Toxicology Letters

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

p-Cresyl sulfate suppresses lipopolysaccharide-induced anti-bacterial immune responses in murine macrophages *in vitro*

Takahiro Shiba*, Ikuyo Makino, Koji Kawakami, Ikuo Kato, Toshihide Kobayashi, Kimiyuki Kaneko

Yakult Central Institute, 5-11 Izumi, Kunitachi-Shi, Tokyo 186-8650, Japan

HIGHLIGHTS

- *p*-Cresyl sulfate (pCS) decreased nitric oxide production in RAW264.7 cells.
- pCS suppressed IL-12 p40 and increased IL-10 production in RAW264.7 cells.
- pCS suppressed lipopolysaccharide-induced CD40 expression on RAW264.7 cells.
- pCS suppressed IL-12 p40 and p70 and increased IL-10 in peritoneal macrophages.

ARTICLE INFO

Article history:
Received 14 October 2015
Received in revised form 11 December 2015
Accepted 13 January 2016
Available online 16 January 2016

Keywords:
p-Cresyl sulphate
RAW264.7 cells
Peritoneal macrophages
Cytokine
Nitric oxide

ABSTRACT

p-Cresyl sulfate (pCS) is a known uremic toxin that is metabolized from *p*-cresol produced by intestinal bacteria. Abnormal accumulation of pCS in the blood is a characteristic of chronic kidney disease (CKD). pCS is suggested to cause immune dysfunction and increase the risk of infectious diseases in CKD patients. In this study, we focused on the effects of pCS on macrophage functions related to host defense. We evaluated the effects of pCS on cytokine production, nitric oxide (NO) production, arginase activity, expression of cell-surface molecules, and phagocytosis in the macrophage-like cell line, RAW264.7. pCS significantly decreased interleukin (IL)-12 p40 production and increased IL-10 production. pCS also decreased NO production, but did not influence arginase activity. pCS suppressed lipopolysaccharide-induced CD40 expression on the cell surface, but did not influence phagocytosis. We further assessed whether the effects of pCS observed in the macrophage-like cell line were consistent in primary macrophages. Similar to RAW264.7 cells, pCS decreased IL-12 p40 and p70 production and increased IL-10 production in primary peritoneal macrophages. These data indicate that pCS suppresses certain macrophage functions that contribute to host defense, and may play a role in CKD-related immune dysfunction.

1. Introduction

p-Cresyl sulfate (pCS) is a uremic toxin that is metabolized from *p*-cresol produced by intestinal bacteria. pCS abnormally accumulates in the blood of patients with chronic kidney disease (CKD), and its toxic effects have been revealed in recent studies. For example, pCS increases reactive oxygen species (ROS) production by enhancing NADPH oxidase activity in renal tubular cells, and induces inflammatory cytokines involved in renal fibrosis (Watanabe et al., 2013). pCS induces muscle insulin resistance accompanied by a defect in the IRS/PI3K/Akt pathway (Koppe et al., 2013), and induces ROS production in endothelial and smooth muscle cells by increasing NADPH oxidase expression (Watanabe et al., 2015). Therefore, pCS is involved in the renal tissue injury, insulin resistance, and endothelial dysfunction in CKD patients. Furthermore, we have noted the involvement of pCS in CKD-related immune dysfunction and consequent risk of infectious diseases. De Smet et al. (2003) showed that serum protein unbound *p*-cresol (including both the conjugated

Abbreviations: APC, allophycocyanin; CKD, chronic kidney disease; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; mAb, monoclonal antibody; MFI, mean fluorescence intensity; NK cells, natural killer cells; NO, nitric oxide; pCS, *p*-cresyl sulfate; PBS, phosphate-buffered saline; PI, propidium iodide; ROS, reactive oxygen species.

* Corresponding author. Fax: +81 42 577 3020.
E-mail address: takahiro-shiba@yakult.co.jp (T. Shiba).

http://dx.doi.org/10.1016/j.toxlet.2016.01.009
0378-4274/

and un-conjugated forms) concentrations were elevated in hemodialysis patients hospitalized for infectious diseases. This clinical study suggested that an elevated pCS concentration contributes to immune dysfunction and the consequent development of infectious diseases. Although several *in vitro* studies have focused on the relationship between *p*-cresol and immune dysfunction, the finding that pCS is a major metabolite in human blood shifted research attention from *p*-cresol to pCS (Vanholder et al., 2011). However, there have been few *in vitro* studies on the role of pCS in immune function (Schepers et al., 2007; Viaene et al., 2012). Recently, we reported that pCS decreased the interferon (IFN)-γ production of T cells and Th1-type cellular immune responses (Shiba et al., 2013). This study mainly focused on the effects of pCS on T cells; thus, the influence of pCS on other types of immune cells remains unknown.

Macrophages play a critical role in the protective immunity against microorganisms (Benoit et al., 2008). Macrophages recruited to the infectious site engulf pathogens by phagocytosis and produce large amounts of pro-inflammatory mediators such as nitric oxide (NO) and cytokines, including interleukin (IL)-12 p70 and tumor necrosis factor (TNF)-α. IL-12 p70 induces IFN-γ from natural killer (NK) cells or activated T cells, and IFN-γ activates cellular immune responses. Antigen presentation by major histocompatibility complex class II and co-stimulatory molecules, including CD80, on macrophages activates T cells and induces the acquired immune response. These diverse anti-bacterial functions of macrophages are promoted by bacterial components such as lipopolysaccharide (LPS). In addition, activated T cells also activate macrophage functions by the cytokine production, including IFN-γ, and the CD40–CD154 interaction in the acquired immune response. Furthermore, impairment of macrophage functions is thought to cause the immune dysfunction of CKD patients and increase susceptibility to infection (Ruiz et al., 1990).

Some uremic toxins were reported to influence macrophage function. For example, phenylacetic acid inhibits inducible nitric oxide synthase (iNOS) expression and nitrate production, and reduces the cytotoxicity of intracellular bacteria (Schmidt et al., 2008). In contrast, indoxyl sulfate stimulates macrophage function and enhances the inflammatory response associated with LPS (Adesso et al., 2013). Guanidino compounds exert proinflammatory as well as anti-inflammatory effects on monocyte/macrophage function (Glorieux et al., 2004). Although the effects of different uremic toxins are distinct, these studies suggest that macrophages are one of the targets. We previously reported that *p*-cresol inhibits IL-12 production by murine macrophages stimulated with a bacterial immunostimulant; however, the effects of pCS on macrophages remain unknown (Kawakami et al., 2009).

In this study, we focused on the effects of pCS on macrophage functions related to host defense. These data should help to elucidate the role and underlying mechanism of pCS in CKD-related immune dysfunction.

2. Material and methods

2.1. Direct cytotoxicity of pCS in RAW246.7 cells

The cytotoxicity of pCS to macrophages was evaluated in a macrophage-like cell line, RAW246.7 (ATCC, Rockville, MD), which was cultured in RPMI 1640 medium (Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 50 μM mercaptoethanol. The RAW264.7 cells (5×10^5 cells/tube) were cultured with or without pCS (0–1000 μM; Tokyo Chemical Industry, Tokyo, Japan) for 24 h. After the treatment, the cells were washed, resuspended in annexin V-binding buffer (BioLegend, San Diego, CA) containing

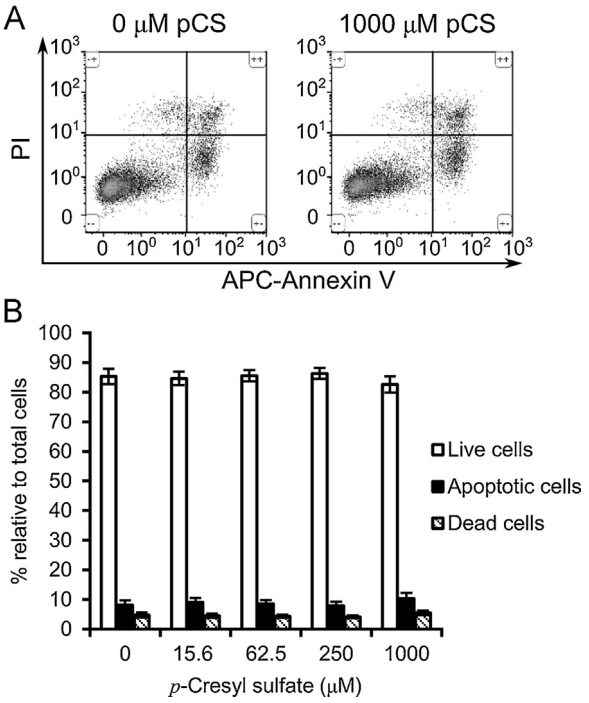

Fig. 1. Cytotoxicity assay of pCS using annexin V and PI staining in RAW264.7 cells. RAW264.7 cells were cultured with or without pCS for 24 h, and then stained with APC-labeled annexin V and PI, and analyzed by flow cytometry. (A) Representative plots gated on single cells. (B) The percentage of live, apoptotic, and dead cells. The data represent mean ± SEM in three independent experiments ($n = 6$). Statistical significance of pCS treatments compared to 0 μM pCS was determined using Dunnett's test.

allophycocyanin (APC)-labeled annexin V (BioLegend) and 1 μg/ml propidium iodide (PI; Sigma–Aldrich, St Louis, MO), and incubated for 15 min. Cells were analyzed with a FACSVerse™ flow cytometer (BD Biosciences, San Diego, CA). Cells negative for both PI and annexin V staining were regarded as live cells; PI-negative/annexin V-positive staining cells were regarded as early apoptotic cells; and PI-positive/annexin V-positive staining cells were regarded as dead cells, including the late stages of apoptotic and necrotic cells. The data were analyzed using Kaluza analysis software (Beckman Coulter, Miami, FL).

2.2. Cytokine production measured by enzyme-linked immunosorbent assay (ELISA)

RAW264.7 cells (1×10^5 cells/well) were seeded in 96-well plates and cultured with 100 ng/ml LPS (Sigma–Aldrich) or 100 ng/ml LPS + 0.2 ng/ml IFN-γ (Peprotech, Rocky Hill, NJ) in the presence or absence of pCS (0–1000 μM). The culture supernatants were collected at 24 h and stored at −80 °C. To determine cytokine concentrations (IL-12 p40, IL-10, and TNF-α) in the culture supernatants, DuoSet ELISA kits (R&D Systems, Minneapolis, MN) were used according to the manufacturer's instructions.

2.3. NO production measured by the Griess assay

The Griess assay was performed as previously described (Munder et al., 1998). Briefly, RAW264.7 cells (1×10^5 cells/well) were seeded in 96-well plates and cultured with 100 ng/ml LPS or 100 ng/ml LPS + 0.2 ng/ml IFN-γ in the presence or absence of pCS (0–1000 μM). After 24-h culture, the culture supernatants were collected. One hundred microliters of the culture supernatant was mixed with 100 μl of Griess reagent [2% sulfanilamide (Sigma–Aldrich) and 0.2% *N*-(1-naphthyl) ethylenediamine