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Original article

Cystine improves survival rates in a LPS-induced sepsis mouse model☆

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SUMMARY

Background & aims: The control of inflammation is important for suppressing severe sepsis. Oral administration of cystine and theanine have been shown to suppress inflammatory responses due to invasion. Furthermore, the uptake of cystine into monocytes is promoted by exposure to lipopolysaccharide (LPS). In the present study, the effects of cystine were examined in the context of inflammatory responses.

Methods: Cystine was orally administered to mice, and the levels of interleukin (IL)-6 in the blood and spleen and the survival rates were calculated after the administration of LPS. The effects of cystine as well as neutralising anti-IL-10 antibodies on the LPS-induced production of IL-6 and IL-10 were examined in a monocyte cell line.

Results: The oral administration of cystine reduced IL-6 levels in the blood and spleen after LPS stimulation and improved survival rates. The addition of cystine to monocytes suppressed LPS-induced IL-6 production but enhanced IL-10 production. A neutralising anti-IL-10 antibody eliminated the inhibitory effects of cystine on the LPS-induced production of IL-6.

Conclusions: The oral administration of cystine suppressed IL-6 production following LPS stimulation and improved survival rates in mice with LPS-induced sepsis. The enhanced production of IL-10 by monocytes may be involved in this anti-inflammatory response.

1. Introduction

Localised inflammatory responses rapidly spread throughout the body after invasion, such as with tissue injury and surgery. Excessively invasive surgery activates monocytes, macrophages (Mφ), and neutrophils in the blood, spleen, and liver, which, in turn, produce inflammatory cytokines (including IL-1, -6, and -8 and tumour necrosis factor (TNF)). These cytokines enter the bloodstream and can then be delivered throughout the body, thereby inducing a systemic inflammatory response syndrome (SIRS) [1]. Persistent and excessive inflammatory responses (e.g., SIRS) are also complicated by compromised immune function and infection, which leads to systemic multiple organ failure (MOF) and severe sepsis [1]. Therefore, controlling perioperative inflammation is important to suppress SIRS due to surgery.

We previously demonstrated that the oral administration of cystine and theanine (CT) enhanced immune function in human and mouse experiments [2–6]. Moreover, the oral administration of CT for 5 days before surgery was recently shown to suppress excessive inflammation and promote recovery after surgery in a mouse model of surgery [7]. In addition, a clinical study of patients who underwent distal gastrectomy revealed that postoperative inflammation was significantly lower following the oral ingestion of CT for 10 days (5 days before and after surgery) compared with placebo [8]. However, the mechanisms responsible for the anti-

Abbreviations: GSH, glutathione; CT, cystine/theanine; IL, interleukin; TNF, tumour necrosis factor; V, vehicle; SIRS, systemic inflammatory response syndrome; MOF, multiple organ failure; ROS, reactive oxygen species; Mϕ, macrophage; LPS, lipopolysaccharide; MAPK, MAP kinase; HPLC, high-performance liquid chromatography; RT-PCR, reverse transcriptase-polymerase chain reaction; TLR, toll-like receptor; FBS, foetal bovine serum.

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inflammatory effects of CT ingestion have yet to be elucidated in detail.

Cystine, one of the sulphur-containing amino acids, is abundant in soybeans, wheat and meat. It is a precursor to glutathione (GSH), which protects cells from the reactive oxygen species (ROS) that are generated by inflammation in all mammalian tissues, especially in the liver [9]. The plasma cystine level is generally varied by food intake and regulated by protein catabolism in peripheral tissues; for example, cystine is supplied to the plasma from peripheral tissues such as skeletal muscle tissues [9]. Cystine plays an important role in the immunological response of Mφs and dendritic cells [9,10]. Extracellular cystine is incorporated into cells through xCT, a transporter on the cell membrane, and is then rapidly metabolised to cysteine [11]. xCT is expressed in monocytes, Mφ, and neutrophils [11,12]. According to previous studies, the expression of xCT in monocytes stimulated with lipopolysaccharide (LPS) was significantly increased within several hours and facilitated the uptake of cystine into cells [13–15]. Thus, excessive inflammation (e.g., SIRS) increases the requirement for cystine by monocytes such that the supply of cystine may be important for controlling excessive inflammation.

In the present study, we investigated the effects of cystine under an excessive inflammatory response due to sepsis, a type of SIRS, using an LPS-induced mouse sepsis model and a monocyte cell line. The effects of a single oral administration of cystine on the production of inflammatory cytokines after LPS stimulation and survival were analysed using an LPS-induced mouse sepsis model. In addition, to elucidate the anti-inflammatory mechanism of action of cystine, the effects of cystine on LPS stimulation were examined using a monocyte cell line expressing the cystine transporter xCT [16].

2. Materials and methods

2.1. Animals

Nine-week-old female BALB/c mice were purchased from Charles River Japan (Yokohama, Japan). The mice were reared under specific pathogen-free (SPF) conditions on a 24-h light–dark cycle and allowed free access to food and water. All experiments were started when the mice reached 12 weeks of age. The mice were fed CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan), a standard laboratory diet comprising the sulphur-containing amino acids L-methionine (0.45 g/100 g diet) and L-cystine (0.35 g/100 g). All animal experiments were conducted after approval by the Ethical Commission for Animal Research of Ajinomoto Co., Inc.

2.2. Cystine administration

Mice were orally administered with cystine (Ajinomoto Co., Inc., Tokyo Japan), which was suspended in 0.5% methylcellulose (Wako, Tokyo Japan), at 10 ml/kg body weight [7]. The control (vehicle) group received 0.5% methylcellulose orally (10 ml/kg). As shown in the supplemental data, mice were also orally administered cystine and theanine (Taiyokagaku Co., Ltd., Yokkaichi, Japan) (cystine: theanine = 5:2, 10 ml/kg of body weight) that was suspended or dissolved in a 0.5% methylcellulose solution using a previously described method [7]. The oral administration was conducted in the morning.

2.3. Analysis of the sepsis model

To assess the production of IL-6 in the blood and spleen, mice received a single oral administration of vehicle or cystine (25, 50, 100, 200 mg/kg), followed by an intraperitoneal administration of 100 μg of LPS (*Salmonella typhimurium*, Sigma, MO, USA) (4–5 mg/kg) two hours later. After the LPS administration, laparotomy was performed under isoflurane anaesthesia, followed by blood sampling and splenectomy. Blood was collected from the inferior vena cava using a 1-ml syringe into a serum separation tube (BD Microtainer®, BD Bioscience, NJ, USA). The collected blood was allowed to stand for 30 min at room temperature, followed by serum separation by centrifugation (10,000 × g, 1 min). The sera and spleen were stored at −80 °C until use.

In the survival study, mice were orally administered vehicle and cystine (100, 200 mg/kg) once daily for five days until the LPS stimulation. Mice were intraperitoneally administered with LPS (5 mg/kg) two hours after the last oral administration. As shown in the supplemental data, mice were also orally administered a vehicle or CT (cystine 200 mg/kg and theanine 80 mg/kg) and they were intraperitoneally administered LPS in a similar manner. The survival rates of the mice were determined by observations every 12 h for 72 h after the administration of LPS.

2.4. Analysis of serum IL-6 levels

Serum IL-6 levels were determined using the Amersham Interleukin-6, [(m) IL-6] mouse Biotrak ELISA system (GE Healthcare, NJ, USA), according to the manufacturer's instructions. Briefly, 50 μL of serum was added to 96-well plates pre-coated with the anti-mouse IL-6 antibody, followed by a 2-h incubation at room temperature. The plate was washed three times. Then, a biotinylated antibody was added, followed by a reaction at room temperature for one hour. After the plate was washed three times, streptavidin-HRP was added, followed by a reaction at room temperature for 30 min. The plate was then washed three times, and a 3, 3′, 5, 5'-tetramethylbenzidine substrate solution was added, followed by a reaction at room temperature for 30 min. Stop solution was added to measure the absorbance at 450 nm. The concentrations of the samples were calculated from the standard curve as the serum IL-6 level.

2.5. Quantification of IL-6 mRNA expression

Total RNA was extracted from the spleen using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. cDNA was synthesised with random primers (Amersham Biosciences, NJ, USA) using the Omniscript Reverse Transcription kit (Qiagen, CA, USA). Quantitative PCR was conducted using SYBR Green PCR Master Mix (Applied Biosystems, CA, USA), followed by analysis using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The primers were as follows: mouse IL-6 (GenBank accession no.NM_031168), 5′-TCC AGT TGC CTT CTT GGG AC-3′ (forward) and 5′-GTG TAA TTA AGC CTC CGA CTT G-3′ (reverse); mouse β-actin (GenBank accession no.X03672), 5′-GGC TCC TAG CAC CAT GAA GA-3' (forward) and 5′-ATC TGC TGG AAG GTG GAC AG-3' (reverse). All primers were designed using the free software Primer3Plus [17]. Gene amplification was carried out as follows: 50 °C for 2 min and 95 °C for 10 min, followed by 95 °C for 15 s and 60 °C for 1 min for 40 cycles. Relative gene expression levels were calculated using the expression level of β-actin as an internal control.

2.6. Cell culture

The human monocyte cell line THP-1 was purchased from the American Type Culture Collection (ATCC; No.TIB-202). RPMI-1640 medium, supplemented with foetal bovine serum (FBS; a final concentration of 10%), penicillin (a final concentration of 100 U/ml), and streptomycin (a final concentration of 100 μg/ml), was used as