ELSEVIER

International Immunopharmacology 6 (2006) 234–240

International Immunopharmacology

www.elsevier.com/locate/intimp

Protective effects of antimicrobial peptides derived from the beetle *Allomyrina dichotoma* defensin on endotoxic shock in mice

Yukari Koyama [a,e], Maki Motobu [a], Kenji Hikosaka [a,f], Manabu Yamada [b], Kikuyasu Nakamura [b], Hisako Saido-Sakanaka [c], Ai Asaoka [c], Minoru Yamakawa [c,f], Kenji Sekikawa [d], Hiroshi Kitani [d], Kameo Shimura [a], Yutaka Nakai [e], Yoshikazu Hirota [g,*]

[a] *Department of Epidemiology, National Institute of Animal Health, Tsukuba, Ibaraki, Japan*
[b] *Department of Production Diseases, National Institute of Animal Health, Tsukuba, Ibaraki, Japan*
[c] *Laboratory of Innate Immunity, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan*
[d] *Department of Molecular Biology and Immunology, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan*
[e] *Laboratory of Animal Health and Management, Tohoku University, Sendai, Miyagi, Japan*
[f] *Institute of Agriculture and Forestry, University of Tsukuba, Tsukuba, Ibaraki, Japan*
[g] *Laboratory of Veterinary Hygiene, Department of Veterinary Medicine, Faculty of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8, Saiwai-cho, Fuchu, Tokyo 183-8509, Japan*

Received 30 May 2005; received in revised form 6 July 2005; accepted 15 August 2005

Abstract

Synthetic peptides, Arg-Leu-Tyr-Leu-Arg-Ile-Gly-Arg-Arg-NH_2 (peptide A) and Arg-Leu-Arg-Leu-Arg-Ile-Gly-Arg-Arg-NH_2 (peptide B), derived from the beetle *Allomyrina dichotoma* defensin, have not only antimicrobial activities but also anti-inflammatory effects by inhibiting tumour necrosis factor-α (TNF-α) production. In the present study, we evaluated the lipopolysaccharide (LPS)-binding activities and the protective effects of these peptides on LPS-induced lethal shock in D-galactosamine (GalN)-sensitized mice. These peptides were shown to bind to erythrocytes coated with LPS and the binding activity of peptide A to LPS was significantly higher than those of peptide B and polymyxin B. Mice were injected intraperitoneally with peptide A or B at doses of 25, 50, 100 and 150 mg/kg before an injection of *Salmonella abortusequi* LPS (5 μg/kg) and GalN (1 g/kg) (LPS + GalN). All of wild-type mice died within 24 h after challenged with LPS + GalN. All of TNF-α-deficient mice challenged with LPS + GalN survived. An injection of peptide A immediately after challenge with LPS + GalN resulted in significantly improved survival rates in a dose dependent manner. Peptide B showed only minor protection. The levels of TNF-α in the ameliorated mice by peptide A were significantly lower than those of challenge control, suggesting a suppressive effect of peptide A on TNF-α production. Furthermore, peptide A-treated mice showed significantly lower levels of asparate aminotransferase and alanine aminotransferase when compared to challenge control. Concordantly, hemorrhage and necrosis in the liver of peptide A-treated mice were less apparent than those of untreated control mice. These results suggest that peptide A has a protective effect on LPS-induced mortality in this mouse model.

Keywords: Synthetic peptide; Lipopolysaccharide; Galactosamine; Tumour necrosis factor-α; Lethal shock

* Corresponding author. Tel./fax: +81 42 367 5776.
E-mail address: hirotayh@cc.tuat.ac.jp (Y. Hirota).

1567-5769/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.intimp.2005.08.008

1. Introduction

Sepsis is associated with the presence of pathogenic microorganisms or their toxins in the blood. It results from infections with either Gram-negative or Gram-positive bacteria. Sepsis may also occur in the absence of detectable bacterial invasion, and endogenous cytokine production has been implicated as initiators and mediators [1,2].

Though antibiotics are used as medicine against bacterial infection, they can stimulate the release of an excess amount of a bacterial outer membrane component, endotoxin [3]. The released endotoxin activates macrophages, endothelial cells and fibroblasts to produce and release potent inflammatory mediators including tumour necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6 and nitric oxide [4].

The antimicrobial peptides are active against a wide range of microorganisms including Gram-positive and Gram-negative bacteria, fungi and yeast [5]. These peptides, an evolutionarily ancient component of the innate immune system [6], can block endotoxin-induced tumour necrosis factor-α (TNF-α) production and reduce the mortality in a septic shock mouse model [7,8].

We demonstrated that synthetic cationic antimicrobial peptides, based on the amino acid sequence of the beetle *Allomyrina dichotoma* defensin, were effective against both Gram-positive and Gram-negative bacteria including antibiotic-resistant pathogenic bacteria [9–11]. Especially, both Arg-Leu-Tyr-Leu-Arg-Ile-Gly-Arg-Arg-NH_2 (peptide A) and Arg-Leu-Arg-Leu-Arg-Ile-Gly-Arg-Arg-NH_2 (peptide B), indicated strong antimicrobial activity and suppressed TNF-α mRNA expression [10]. Furthermore, we reported that the supplement of these synthetic peptides to macrophages cultured with LPS resulted in a significant decrease in nitric oxide and TNF-α production [12], suggesting their suppressive effects on LPS-induced macrophages activation. In the present study, the protective effects of these peptides on a lethal shock in mice injected with LPS and GalN (LPS + GalN) were investigated.

2. Materials and methods

2.1. Animals

BALB/c female mice (5 weeks of age) and C57BL/6 male and female mice (5–10 weeks of age) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and CLEA Japan, Inc.(Tokyo, Japan), respectively. TNF-α-deficient mice (5–10 weeks of age) with C57BL/6 genetic background were generated in our institute. TNF-α-deficient mice bred in the institute's small animal breeding unit by mating heterozygous parents, and their genotypes were confirmed by PCR amplification [13,14]. TNF-α-deficient mice developed normally and were fertile. All mice were kept in an air-conditioned room and fed standard laboratory food pellets and water ad libitum. This study was done according to the Guideline for Animal Experiment of National Institute of Animal Health, Japan.

2.2. Peptides synthesis

Two peptides used in this study were designed and synthesized using the methods described in the previous study [10]. Briefly, 9-Fluorenylmethoxycarbonyl (F-moc) L-amino acids and F-moc-tris (alkoxy)-benzylamide-poly (ethylene glycol) polystyrene resins were purchased from PE Biosystems, (California, USA). The 9-mer peptides, peptide A and B, were synthesized by a solid-phase method in a 9050 Plus Peptide synthesizer (PerSeptive Biosystems California, USA). Each peptide was purified to homogeneity by an AKTA Explorer using a reverse-phase column of Resource RPC (3 ml) (Pharmacia). The column was eluted for 20 min at 2 ml/min, using a linear gradient from 0% to 30% (v/v) of acetonitrile in water containing 0.05% (v/v) trifluoroacetic acid. Amino acid sequence and molecular mass of peptides were confirmed by a protein sequencer (Procise™ cLC, PE Biosystems California, USA) and matrix-assisted laser desorption ionization mass spectrometry (Voyager, PerSeptive Biosystems), respectively.

2.3. Erythrocyte agglutination assay

One milliliter of 0.5% sheep erythrocytes was sensitized by incubation with 0.2 ml of Re-LPS (*Salmonella Minnesota* R595, Sigma, St. Louis, MO, USA) solution (100 μg/ml in phosphate-buffered saline [PBS]) for 30 min at 37 °C, followed by washing PBS [15]. Fifty microliters of a 0.5% suspension of sensitized erythrocytes was mixed with 50 μl of a twofold serial dilution of peptide A, peptide B and polymyxin B (Wako Pure Chemical, Osaka, Japan) in U-bottom microtiter plates, and then incubated at 37 °C for 1 h. Agglutinin titers were expressed as the mean $\log_2$ of the reciprocal of the highest dilution giving 50% agglutination.

2.4. LPS challenge and survival analysis

Intraperitoneal injection of *Salmonella abortusequi* LPS (5 μg/kg, Sigma, St. Louis, MO, USA) and GalN (1 g/kg, Wako Pure Chemical, Osaka, Japan) induced 100% lethality in 5-week-old female BALB/c mice and 5 to 10-week-old male and female C57BL/6 mice, and this protocol was used as an endotoxic shock model. BALB/c mice were divided into 13 groups, as shown in Table 1. Group 1 was a negative control group without any treatment. Group 2 was a challenge control group injected with LPS + GalN. Groups 3, 4 and 5 were injected intraperitoneally with peptide A at the doses of 50, 100 and 150 mg/kg, immediately after injection with LPS + GalN. Groups 6, 7 and 8 were injected with peptide A alone at the doses of 50, 100 and 150 mg/kg. Groups 9, 10 and 11 were injected with peptide B at the doses of 25, 50 and 100 mg/kg, immediately