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Short Report

Heat-killed *Propionibacterium Acnes* Up-regulates Toll-like Receptor 4 on Macrophages

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Abstract

It is known that heat-killed *Propionibacterium acnes* (*P.acnes*) induces lipopolysaccharide (LPS) hypersensitivity. However, effect of the *P.acnes* on expression of Toll-like receptor (TLR) 4 remains unclear. To determine whether or not TLR4 expression of macrophages can be changed by the *P.acnes* stimulation, TLR4 of RAW264.7 cells as measured after the *P.acnes* stimulation in vitro. Furthermore, the *P.acnes*-treated mice were measured as to the expression of TLR4 mRNA in the liver. The *P.acnes* treatment increased the TLR4 expression on RAW264.7 cells. The *P.acnes*-treated mice also were expressed as TLR4 mRNA in the liver. These results may suggest that an up-regulation of TLR4 on macrophages can be induced by heat-killed *P.acnes* treatment.

Introduction

Biological response modifiers (BRM) induce an activation of monocytes- macrophages, which results in increased production of cytokines and chemical mediators [1]. Indeed, lipopolysaccharide (LPS), a BRMs, increased production of interleukin (IL)-1, tumor necrosis factor (TNF) and prostaglandin (PG) in Kupffer cells [2], which are hepatic macrophages. Shuto Y., et al. [3] reported that under an experimental treatment by heat-killed *Propionibacterium acnes* (*P.acnes*) gram-positive bacteria enhances lipopolysaccharide (LPS) hypersensitivity. Therefore, severe liver damage and septic shock were caused by LPS injection in the *P.acnes*-sensitized mice [4]. Although it was reported that the *P.acnes*-induced LPS-dependent hypersensitivity mediated interferon (IFN) -γ [5], it is possible that excessive expression of Toll-like receptors (TLR) 4, which is the main receptor of LPS [6], can be induced by the *P.acnes* stimulation. However, the effect of the *P.acnes* stimulation on the TLR4 expression is poorly understood. In this study, we investigated to determine whether or not TLR4 expression of macrophages can be changed by a treatment of heat-killed *P.acnes*.

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Materials and Methods

Cell-line and animals

Propionibacterium acnes was purchased by Laboratory of Culture Collection, University of Tokyo. The bacteria were cultured for 2 days under anaerobic conditions in a brain-heart infusion, harvested, killed by treatment at 60 ℃ for 60 min, and freeze-dried.

RAW264.7 cells, derived from a mouse macrophage cell-line (Riken), were plated in a 24-well plate at a concentration of 1 x 10^6 cells/well and incubated with DMEM containing 10% FCS in a CO2 incubator (37 ℃ and 5%CO_2).

Male C3H/HeN mice (19-24g, Clea Japan) were housed in a controlled environment (20±1 ℃, 12:12-h light-dark cycle) and were maintained ad libitum on diets and water. The experiment followed the guideline set forth in the Care and Use of Animals in the Field of Physiological Science approved by the Council of Physiological Society of Japan.

Measurement of TLR4 expression on RAW 264.7 cells

RAW264.7 cells were stimulated by heat-killed *P.acnes* (100 g/ml) in each well. After incubation, the cells were collected, and then stained for phycoerythin (PE)-conjugated anti-CD14, fluorescein isothiocyanate (FITC)-conjugated anti-TLR4/MD-2. Cells were analyzed by flow cytometry (FACScan with CellQuest software, Becton & Dickinson). Specificity of the staining was confirmed by comparing to results stained with nonspecific isotype antibodies for control.

Effect of *P.acnes* treatment on TLR4 mRNA expression, infiltration of inflammatory cells in the liver and on LPS induced animal motility

The RNA was extracted from the liver, which was removed from mice on 0, 1, 3 and 7th days after the *P.acnes* treatment (750mg/kg by i.p.) and it was reverse transcribed, and then the cDNA was added to a polymerase chain reaction (PCR), in the mixture of which Taq polymerase and each primer (β-actin : forward ; 5'-TAA AAC CCA GCT CAG TAA CAG TCC G, reverse ; 5'-TGG AAT CCT GTG GCA TCC ATG AAA C, TLR4 : forward ; 5'-AGT GGG TCA AGG AAC AGA AGC A, reverse ; 5'-CTT TCA CAG CTC ATT TCT CAC C). Each amplification cycle included denaturation at 94 ℃ 30 sec, annealing for 30 sec at 55 ℃, and extension for 30 sec at 72 ℃. The amplification cycle was repeated 30 times. Amplified products were identified by ultraviolet illumination after electrophoresis of the PCR products in a 1.2% agarose gel containing ethidium bromide.

To examine inflammatory cells infiltrations in the liver, the mouse liver was fixed by 10% neutral-buffered formaldehyde seven day after the *P.acnes* treatment (750mg/kg by i.p.). Then the fixed tissues were embedded in paraffin. The specimens were stained with hematoxylin and eosin (H&E).

To examine an additional effect of heat-killed *P.acnes* on LPS hypersensitivity, survival was monitored over the 24 h after the *P.acnes*- (n=5) or PBS- (n=5) treated mice were injected i.v. with LPS, 1mg/kg.

Results and Discussion

It is known that IFN-γ is one of the important mediators for hypersensitivity to LPS induced by *P.acnes* [5]. IFN-γ seems to be produced by NK and/or T cells after the LPS challenge [7]. However, functional changes of macrophages in this process remain unclear. To examine the possible changes in TLR4 expression on the surface of macrophages induced by heat-killed *P.acnes* treatment, we performed flow cytometry of RAW264.7 cells (Fig. 1). In the normal control (PBS treatment), TLR4$^+$/CD14$^+$ cells ratio as only 1.66% (Fig.1A). In addition, the expression level did not change after the LPS challenge (1.08%, Fig. 1B). We found, however, that both cells increased 18.75% and 23.70% in ratio, respectively, 24h and 48h after the *P.acnes* stimulation (Fig. 1C and E), and their expression levels were little affected by the LPS challenge (Fig.1D and F).

From these findings it may be reasonable to assume that the TLR4 expression on macrophages can be accelerated by the *P.acnes* stimulation in vitro, and that such qualitative change of macrophages may participate in increasing the LPS sensitivity after the *P.acnes* treatment. The present findings may be indirectly supported by the previous study [8], indicating in vivo mouse experiments that the hypersensitivity to LPS by the *P.acnes* is not CD14 dependent.

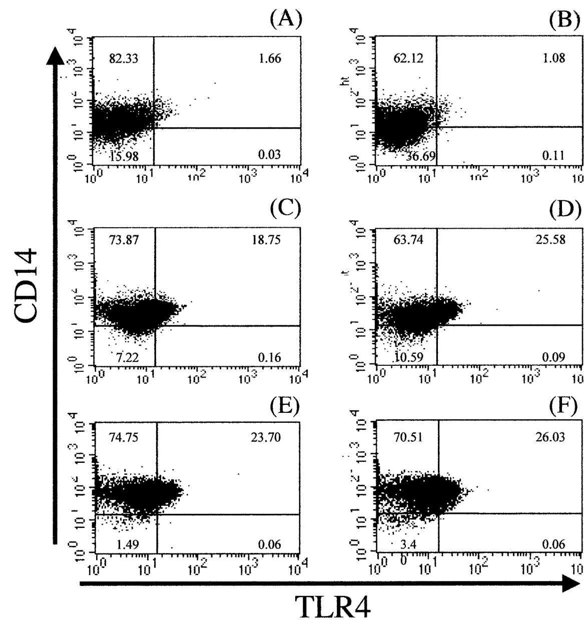

Fig. 1 In vitro effect of heat-killed *P.acnes* on cell surface expression of TLR4 and CD14 in macrophage cell-line, RAW 264.7 cells, before and after LPS stimulation. The cells were stimulated with PBS (A and B) and heat-killed *P.acnes* (100μg/well) for 24h (C and D) and 48h (E and F) at 37°C. B, D and F showed 6h after LPS stimulation. After the stimulation, the cells were stained with the anti-TLR4/MD-2 (FITC-conjugated) antibody and the anti-CD14 (PE-conjugated) antibody. Percentages are shown in each quadrant. This is representative data from the 3 experiments performed.

In addition, we studied the expression of TLR4 mRNA in the liver of mice after the treatment of *P.acnes*, because hepatic macrophages (Kupffer cells) represent the largest group of macrophages in whole body [9]. The expression level of TLR4 mRNA increased day by day on mice after the *P.acnes* treatment (Fig. 2A). Although we did not examine the TLR4 mRNA expression of hepatic macrophages in vivo, this finding may suggest that the TLR4 can be induced by the challenge of heat-killed *P.acnes*.

A histochemical study revealed characteristic granulomas in the liver at 7th day after injection of the *P.acnes* (Fig. 2B). It is already reported as a model of fulminant hepatitis [4] that infiltration of monocytes

and Kuppfer cells have reached to the primed stage at 7th day after the *P.acnes* injection, and thereafter overproduction of inflammatory cytokines from the primed macrophages is induced by a particle of LPS stimulation [5,10]. We observed remarkable decreases of survival rate in the *P.acnes*/LPS experiment group (Fig. 2C).

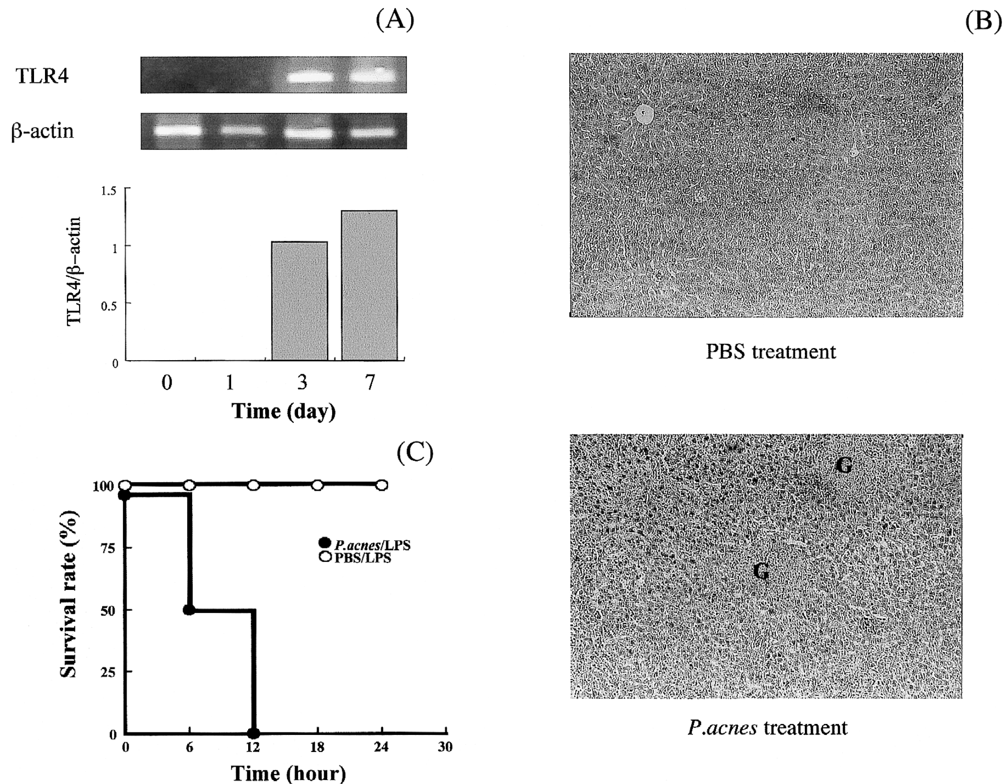

Fig. 2 Effect of heat-killed *P.acnes* injection on mouse liver damage and mortality. (A) Time courses of induction of TLR4 mRNA in the liver from the *P.acnes* treatment were shown. The expression of TLR4 mRNA was determined by RT-PCR. The normalized levels of TLR4 mRNA were shown as relative β-actin expression. (B) Histological features of the C3H/HeN mouse liver 7th day after i.p. injection of the heat-killed *P.acnes* or PBS were shown. The liver sections were stained with H & E (Original magnification $\times$ 25). (C) Involvement of the *P.acnes* in LPS-induced mortality of C3H/HeN mice was shown, G ; granulomas. The mice were pretreated with 750μg of heat-killed *P.acnes* or Saline. Seven days later, 10μg of LPS was injected into the mice, and the mortality of the mice was followed up. Each group consisted of 5 mice.

The present results may suggest that an up-regulation of TLR4 on macrophages is induced by heat-killed *P.acnes* treatment, and that its up-regulation is necessary at least for LPS hypersensitivity, such as in sepsis syndrome and Shwartzman reaction [11]. [Grant from Ministry of Education, Science, Sports and Culture of Japan (163500433): to H.Yano].

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