

TUMOUR necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a major pro-inflammatory cytokine inducing the synthesis and release of many inflammatory mediators. It is involved in immune regulation, autoimmune diseases, and inflammation. Our previous study demonstrated that acanthoic acid, (-)-pimara-9(11), 15-dien-19-oic acid, a pimaradiene diterpene isolated from *Acanthopanax koreanum*, inhibited TNF- $\alpha$  production. To extend our understanding of inhibitory effects of acanthoic acid on TNF- $\alpha$  production, its effects on TNF- $\alpha$  gene expression was tested. Based on the results from RT-PCR and promoter analysis of TNF- $\alpha$ , it was found that acanthoic acid suppressed TNF- $\alpha$  gene expression. But the same concentration of acanthoic acid had no effect on IL-6 gene expression. Haptoglobin is an acute phase protein which is induced by TNF- $\alpha$ . When liver cells were treated with acanthoic acid, haptoglobin synthesis was blocked by acanthoic acid. These data confirmed that acanthoic acid inhibited gene expression and biological function of TNF- $\alpha$ .

**Key words:** Acanthoic acid, TNF- $\alpha$ , Haptoglobin

## Effects of acanthoic acid on TNF- $\alpha$ gene expression and haptoglobin synthesis

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## Introduction

Tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) is produced by many different types of cells including activated macrophages and fibroblasts. It has diverse effects on the target cells including inducing cytotoxicity to L929 cells, stimulating fibroblast cell proliferation, and increasing PGE<sub>2</sub> and arachidonic acid production.<sup>1-3</sup> TNF- $\alpha$  is also involved in many immune diseases such as rheumatoid arthritis,<sup>4</sup> sepsis, immunodeficiency,<sup>5</sup> insulin-dependent diabetes mellitus<sup>6</sup> and inflammation. The regulation of TNF- $\alpha$  production is critical in the maintenance of homeostasis of the immune system and in the prevention and treatment of the immune diseases.

Acanthoic acid, (-)-pimara-9(11), 15-dien-19-oic acid, is a diterpene isolated from the root bark of *Acanthopanax koreanum*, which has been traditionally used as a tonic and sedative as well as in the treatment of rheumatism and diabetes in Korea.<sup>7</sup> Previous studies<sup>8,9</sup> using this diterpenoid compound demonstrated that it inhibited TNF- $\alpha$  production by human monocytes/macrophages and in the sera of silicosis model. Also, its oral administration into the mice significantly reduced the writhing syndrome induced by acetic acid and it reduced leukotriene B<sub>4</sub> production by leukocytes *in vitro* at the concentration of about 30  $\mu$ g/ml. In this study, to analyse the effects of acanthoic acid on TNF- $\alpha$  production further, we tested it on TNF- $\alpha$  gene expression and haptoglobin production regulated by TNF- $\alpha$ .

## Materials and Methods

### Preparation of acanthoic acid

Acanthoic acid was isolated essentially as described previously.<sup>7</sup> Usually, more than 95% pure acanthoic acid was obtained by fractionation on a silica-gel column chromatography (*n*-hexane-ethylacetate, 20:1 to 5:1).

### Isolation of human monocytes/macrophages

Heparinized blood obtained from healthy donors was overlaid on a Ficoll-Hypaque density gradient and centrifuged at 700  $\times$  g for 30 min. Mononuclear cells and neutrophils were obtained at the density of 1.077 g/l and of 1.077–1.119 g/l respectively. Mononuclear cells were incubated in a 24-well culture plate for 2 h and adherent monocytes/macrophages (>90% purity by non-specific esterase staining) were obtained. Then the cells were preincubated with various concentrations of acanthoic acid for 1 h and stimulated with 100  $\mu$ g/ml silica. After 48 h incubation, the culture supernatants were assayed for TNF- $\alpha$  using a TNF- $\alpha$  ELISA kit obtained from Genzyme (Cambridge, MA).

### Transfection of TNF- $\alpha$ CAT construct

HeLa cells ( $5 \times 10^5$  cells/sample) were transfected with 10  $\mu$ g TNF- $\alpha$  promoter-CAT reporter plasmid

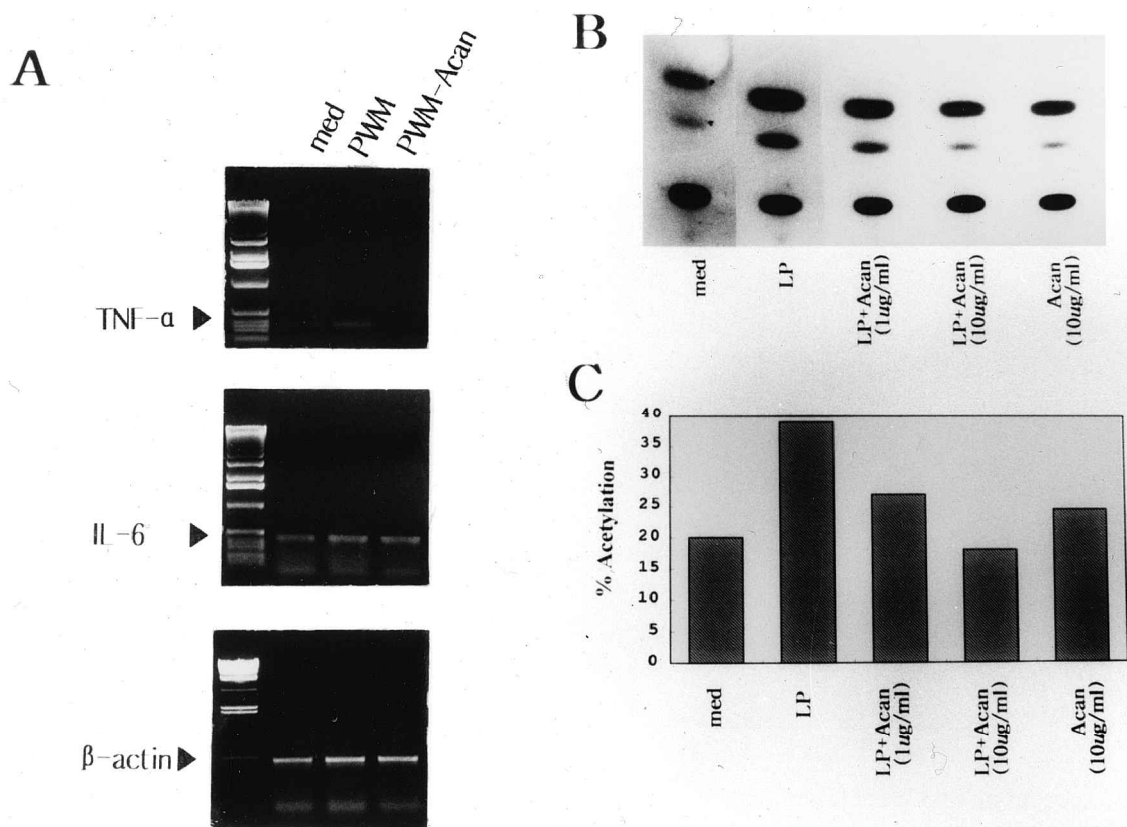


FIG. 1. Acanthoic acid inhibited TNF- $\alpha$  gene expression (A) Human monocytes/macrophages ( $1 \times 10^6$  cells/well) were incubated with  $10 \mu\text{g/ml}$  of acanthoic acid for 1 h and stimulated with  $10 \mu\text{g/ml}$  PWM. Then RT-PCR was performed using specific primers. (B) HeLa cells ( $5 \times 10^5$  cells/sample) were transfected with  $10 \mu\text{g}$  TNF- $\alpha$  promoter-CAT reporter plasmid using calcium-phosphate method. After 16 h incubation, cells were treated with  $10 \mu\text{g/ml}$  LPS plus  $100 \text{ ng/ml}$  PMA for 18 h in the presence or absence of acanthoic acid. Then  $10 \mu\text{g}$  of cell lysates were assayed for CAT activity. (C) The CAT activity was calculated as follows: % acetylation = (cpm in acetylated species/cpm in acetylated species + non-acetylated chloramphenicol)  $\times 100$ .

using calcium-phosphate method. After 16 h incubation, cells were treated with  $10 \mu\text{g/ml}$  LPS plus  $100 \text{ ng/ml}$  PMA for 18 h in the presence or absence of acanthoic acid. Then  $10 \mu\text{g}$  of cell lysates were assayed for CAT activity. The CAT activity was calculated as follows: acetylation rate = (cpm in acetylated species/cpm in acetylated species + non-acetylated chloramphenicol).

## Results and Discussion

Previous results showed that acanthoic acid inhibited TNF- $\alpha$  production from human monocytes/macrophages stimulated with  $100 \mu\text{g/ml}$  silica. Several diterpene compounds having similar structures isolated from the root bark of *Acanthopanax koreanum* were tested for inhibiting TNF- $\alpha$  synthesis. Acanthoic acid significantly inhibited TNF- $\alpha$  production from human monocytes/macrophages ( $73.4 \pm 7.9\%$  inhibition compared with untreated control), but other diterpenes had no effects on TNF- $\alpha$  production (data not shown). It also suppressed TNF- $\alpha$  production in the sera of experimental silicosis rats ( $79.5 \pm 1.4\%$  inhibition compared with untreated silicosis rats).

To know the molecular action of acanthoic acid on TNF- $\alpha$  synthesis, the effects of acanthoic acid on TNF- $\alpha$  gene expression were tested. Acanthoic acid suppressed PWM-induced TNF- $\alpha$  gene expression in human monocytes/macrophages (Fig. 1A). But it had no effects on the gene expression of IL-6 and  $\beta$ -actin. Furthermore, acanthoic acid suppressed the promoter activity of TNF- $\alpha$  activated with LPS and PMA (Fig. 1B,C).  $10 \mu\text{g/ml}$  acanthoic acid inhibited about 50% of LPS and PMA-induced TNF- $\alpha$  promoter activity.

TNF- $\alpha$  is involved in hepatic inflammation and fibrosis. In the liver, TNF- $\alpha$  induces a group of acute phase proteins including haptoglobin.<sup>10</sup> Haptoglobin is related to the status of inflammation, infection and malignancy.<sup>11-13</sup> So we tested the effects of acanthoic acid on haptoglobin synthesis of hepatocytes. TNF- $\alpha$  inhibited haptoglobin synthesis of Hep3B cells stimulated with LPS and PMA (Fig. 2), but it had no effects on Hep3B cell proliferation (data not shown).

Acanthoic acid is a diterpene isolated from *Acanthopanax koreanum*. It has anti-inflammatory effects *in vivo* and *in vitro*. In this study, it is demonstrated

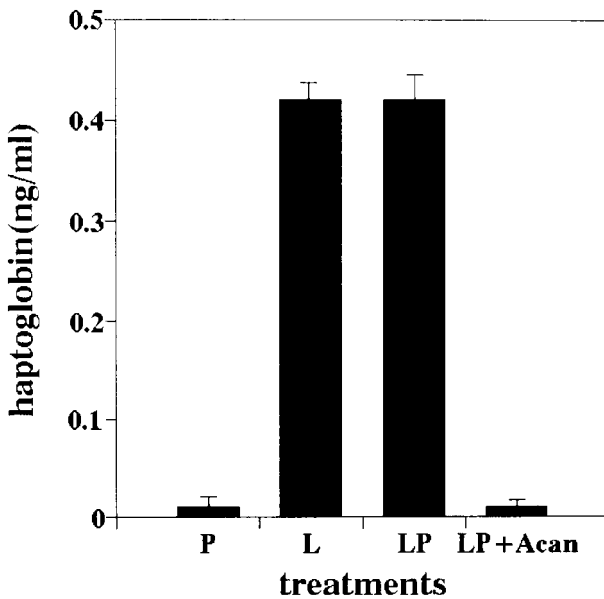


FIG. 2. Acanthoic acid inhibited haptoglobin synthesis from Hep3B cells. Hep3B cells ( $4 \times 10^4$  cells/well) were starved in a serum-free RPMI 1640 medium for overnight at 37°C. Then the cells were washed with serum-free RPMI 1640 medium. The cells were preincubated with 10  $\mu$ g/ml acanthoic acid for 1 h and stimulated with 10  $\mu$ g/ml LPS and/or 100 ng/ml PMA. After 48 h incubation, the culture supernatants were assayed for haptoglobin production using haptoglobin ELISA. Data represent the mean  $\pm$  SD of four different determinants.

that the main intracellular target of acanthoic acid is machinery of TNF- $\alpha$  gene expression. The exact target molecule which interacts with acanthoic acid needs to be defined. Considering its action of mechanism, it will be used as an anti-inflammatory agent to treat many inflammatory diseases.

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