ANTI-TUMOR NECROSIS FACTOR-α ACTIVITY OF DIOSGENIN IN NAEGLERIA FOWLERI LYSATE-INDUCED HUMAN MONOCYTE U937-DERIVED MACROPHAGES

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ABSTRACT

Free-living Naegleria fowleri amoebae cause fatal primary amoebic meningoencephalitis in humans. Additionally, N. fowleri lysate induces strong cytopathic effects and proinflammatory cytokines in microglial cells. Our previous study revealed that diosgenin has an anti-amoebic activity against N. fowleri trophozoites and less toxicity in mammalian cells than amphotericin B. In the present study, we investigated the effect of diosgenin on the gene expression of proinflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 in human monocyte U937-derived macrophages induced by N. fowleri lysate. The results revealed that the maximum non-toxic dose of diosgenin in macrophages was 100 mg/ml (<10% inhibition of proliferation) at 24, 48, and 72 h. The 50% growth inhibition values of N. fowleri lysate in macrophages at 24, 48, and 72 h were 8.361±0.071, 8.383±0.173, and 7.786±0.218 mg/ml, respectively. N. fowleri lysate-induced TNF-α gene expression was increased at 3, 6, and 12 h. Lipopolysaccharide (LPS)-induced TNF-α gene expression was also increased at the same time. Diosgenin decreased TNF-α gene expression in N. fowleri lysate- and LPS-induced cells at 3, 6, and 12 h. No significant difference in IL-1β and IL-6 expression of untreated and diosgenin-treated cells after stimulation with N. fowleri lysate or LPS was observed at 3, 6 and 12 h. In conclusion, diosgenin has an anti-inflammatory activity against human monocytes by blocking TNF-α synthesis.

Keywords: Diosgenin, Naegleria fowleri lysate, proinflammatory cytokine

INTRODUCTION

Naegleria fowleri is ubiquitously distributed worldwide in various warm aquatic environments and soil habitats. N. fowleri amoeba causes primary amoebic meningoencephalitis (PAM) in humans. PAM is a rare but almost always fatal disease of the central nervous system (Schild et al., 2007; Visvesvara et al., 2007; Marciano-Cabral, 2009), which is often reported in healthy children and young adults after exposure to contaminated recreational, domestic or environmental water sources (Visvesvara et al., 2007; Marciano-Cabral, 2009). The mechanisms of contact-dependent N. fowleri pathogenicity were previously observed to proceed via formation of vigorous pseudopodia and food-cup structures by trophozoites incubated with various target cell types (Cho et al., 2003). N. fowleri lysate induces strong cytopathic effects and the release of proinflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 from microglial cells (Lee et al., 2011).

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The role of humoral immunity in the response to *N. fowleri* has been studied in experimental animals and humans. Previous studies have reported that anti-*Naegleria* IgG or an anti-*N. fowleri* monoclonal antibody prolong the time to death by immobilization of amoebae and prevention of their rapid migration to the brain, enhancement of complement-mediated lysis, and neutralization of amoeba cytotoxins (Lallinger et al., 1987). The IgA and IgM in mucosal secretions of humans can prevent amoebic infection by blocking adhesion of trophozoites to mucosal epithelium (Rivera et al., 2001).

Studies of CNS infection (PAM) have found that microglia play an important role in host resistance to *Naegleria* by the production of proinflammatory cytokines IL-1β, IL-6, and TNF-α in the brain (Marciano-Cabral, 2009). Therefore, activated microglia may activate astrocytes and a cascade of inducible inflammatory cytokine expression, resulting in hyperinflammation, breakdown of the blood–brain barrier, and an influx of immune cells from nonneuronal sites (Oh et al., 2005).

Diosgenin, a purified extract of *Momordica charantia*, has a highly potent anti-amoebic activity caused by a decrease in the number of sucker-like apparatuses, inhibition of food cup formation, and down-regulation of the nf cys gene expression of *N. fowleri* trophozoites at 6 and 12 h post-exposure. The toxicity to mammalian cells is less than that of amphotericin B (Rababert et al., 2015). Previous studies have revealed that diosgenin has anti-inflammation effects on human osteoarthritic chondrocytes (Wang et al., 2015) and human THP-1 monocytic cells (Yang et al., 2013).

To date, no viable data have indicated that diosgenin has anti-inflammation effects on human macrophages after stimulation with *N. fowleri* lysate. In the present study, we investigated the effect of diosgenin on the gene expression of proinflammatory cytokines TNF-α, IL-1β, and IL-6 in human monocyte U937-derived macrophages induced by *N. fowleri* lysate.

**MATERIALS AND METHODS**

**Reagents**

Diosgenin was donated by Natchagorn Lumlerdjik, the Center for Applied Thai Traditional Medicine, Faculty of Medicine, Siriraj Hospital, Mahidol University. One gram of diosgenin was dissolved in 5 ml dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO), resulting in a stock solution with a concentration of 200 mg/ml that was stored at −20°C until use. Lipopolysaccharide (LPS) from *Escherichia coli* strain 0127:B8 was purchased from Sigma-Aldrich. One milligram of LPS was dissolved in 8 ml distilled water (Thai Otsuka, Bangkok, Thailand) to give a final concentration of 125 µg/ml that was used as a stock solution (stored at −20°C until use).

**Cell culture**

The human monocyte U937 cell line was donated by Professor Prasert Auewarakul, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University. The cells were grown in RPMI-1640 medium (Gibco-BRL, Grand Island, NY) containing HEPES and supplemented with 10% heat-inactivated fetal calf serum (FCS, Invitrogen, Carlsbad, CA), 2 mM glutamine, and 110 mg/ml sodium pyruvate on 6-well culture plates (Corning, NY) at a density of 5×10⁶ cells/ml as described previously (Ghosh et al., 2010) with some modification. U937 cells were differentiated into macrophage-like cells by treatment with 250 ng/ml phorbol-12 myristate acetate (PMA; Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO₂. The medium was replaced after 4 days of culture.

**N. fowleri lysate**

*N. fowleri* trophozoites (Siriraj strain) were axenically cultured at 37°C in Nelson’s medium supplemented with 5% FCS (Tiewchareon et al., 2014). The trophozoites were harvested by incubation at 4°C for 10 min, scraping, and centrifugation at 500 g (Thermo Scientific, England) for 30 min. The cell pellet was resuspended in pre-chilled RPMI-1640 at a ratio
of 1:4 and incubated on ice for 10 min. The cell suspension was sonicated with 10 short bursts of 10 s each, followed by intervals of 3 min for cooling. Cell debris was removed by centrifugation at 4°C for 30 min at 500 g. The protein concentration was measured by a Nanodrop 1000 spectrophotometer (Thermo Scientific). The *N. fowleri* lysate at a protein concentration of 40 mg/ml was aliquoted and frozen at −80°C until use.

**MTT colorimetric assay**

To evaluate the maximum non-toxic dose (MNTD) of diosgenin, human U937 monocytes (2×10⁵/well) were seeded in 96-well plates containing RPMI-1640 with PMA and cultured for 7 days. Human monocyte U937-derived macrophages were treated with various concentrations of diosgenin (0–4000 mg/ml) for 24, 48, and 72 h. At the indicated times, the cells were washed twice with phosphate-buffered saline (PBS), pH 7.4. The cells were incubated with MTT solution for 2 h before lysis with DMSO. The absorbance was measured after 30 min using a microplate reader (Wallac 1420 Multilabel counter; Perkin Elmer) at a wavelength of 590 nm. Medium containing DMSO (5%) served as the negative control. Results were generated from two independent experiments. Each experiment was performed in triplicate. MNTD values were calculated with Probit analysis software (LdP Line software).

**Cytotoxicity assay**

To evaluate the cytotoxicity of the *N. fowleri* lysate, human monocyte U937-derived macrophages were seeded in 96-well plates at an initial density of 2×10⁵ cells/well in RPMI-1640 supplemented with L-glutamine and 10% FCS in the presence of diosgenin at the MNTD or RPMI-1640 medium alone for 1 h. After pre-incubation with diosgenin or medium alone, the cells were treated with *N. fowleri* lysate (CC₅₀) or LPS (5 mg/ml) (Galdireo et al., 2005) for 3, 6, 12, 24, and 48 h (Lee et al., 2011).

At the indicated times, RNA was extracted from the untreated and diosgenin-treated macrophages induced by *N. fowleri* lysate or LPS using an E.Z.N.A.® Total RNA Kit I (Omega Bio-Tek, Norcross, GA) according to the manufacturer’s instructions. cDNA was generated from 0.2 µg RNA using a Maxime RT PreMix Kit (iNtRON Biotechnology, Kyungki-Do, Korea) and then stored at −20°C until use. The cDNA was diluted at 1:10, and 1 µl of each sample was subjected to PCR amplification with SYBR® Green Master Mix (BioRad). The reaction was carried out using gene-specific primers for TNF-α, IL-1β, IL-6, and a housekeeping gene, b-actin (Table 1) on the Chromo 4 Real-Time System (Bio-Rad). PCR consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 60 s, annealing at 60°C for 30 s, and extension with data collection at 72°C for 60 s. After each run, melting curves were acquired by stepwise increases in temperature from 65°C to 95°C at a heating rate of 1°C per min to ensure that a single product was amplified in the reaction. The threshold cycle (Ct) for each gene of interest and the housekeeping gene (b-actin), and the difference between their Ct values (DCt) were determined. For each gene of interest, normalization was performed against untreated macrophages as the reference, and its DCt value was subtracted from the DCt value of diosgenin-treated macrophages induced by *N. fowleri* lysate or LPS to obtain the DDCt or D value.
Finally, the $2^D$ value was calculated to reflect the relative expression of each selected gene (Livak and Schmittgen, 2001). The PCR product was visualized by electrophoresis for 30 min using 5 µl reaction mixture at 100 V in a 1.8% agarose gel containing 1 µg/ml Gel red. The gels were examined on a Bio-Rad Gel Doc and photographed.

RESULTS

Effects of diosgenin and *N. fowleri* lysate on macrophage proliferation

MTT assays revealed that the MNTD of diosgenin in human monocyte U937-derived macrophages was 100 mg/ml (<10% inhibition of growth) at 24, 48, and 72 h. The 50% cytotoxicity concentration ($CC_{50}$) values of diosgenin at 24, 48, 72 h in macrophages were 904.759±15.723, 906.989±15.5825, and 835.012±14.9315 mg/ml, respectively. In contrast, 1000–4000 µg/ml diosgenin exhibited significant dose-dependent inhibitory effects on macrophages at 24, 48, and 72 h (Figure 1A) with more than 50% suppression.

The $CC_{50}$ values of *N. fowleri* lysate in macrophages were 8.361±0.071, 8.383±0.173, and 8.786±0.218 mg/ml at 24, 48, and 72 h, respectively. *N. fowleri* lysate at 10–40 mg/ml exhibited significant dose-dependent growth inhibition in macrophages (Figure 1B) with more than 70% suppression.

Morphological characteristics of untreated and diosgenin-treated human monocyte U937-derived macrophages induced by *N. fowleri* lysate

The morphological characteristics of human monocyte U937-derived macrophages induced by *N. fowleri* lysate in the absence or presence of diosgenin was observed under an Olympus IX 70 inverted microscope equipped with a digital camera for 72 h.

In medium without PMA, U937 cells had a smooth and round surface (data not shown). After addition of PMA, human monocyte U937-derived macrophages formed cell clusters and extended pseudopodia (Figure 2A), which is in agreement with a study by Garrelds et al (1999). Diosgenin-treated macrophages maintained normal cell morphology (Figure 2B). Macrophages induced by *N. fowleri* lysate underwent severe morphological changes including disruption and breakdown of the plasma membrane and a large round shape (Figure 2C), whereas diosgenin-treated macrophages induced by *N. fowleri* lysate underwent slightly different morphological changes (Figure 2D). As the positive control, LPS-induced macrophages were similar to *N. fowleri* lysate-induced macrophages in the absence (Figure 2E) or presence (Figure 2F) of diosgenin. Our results showed that diosgenin at the MNTD prevented the changes in macrophages induced by *N. fowleri* lysate.

<table>
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<tr>
<th>Gene name</th>
<th>Forward primer (5′→3′)</th>
<th>Reverse primer (5′→3′)</th>
<th>Product size (bp)</th>
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<td>TNF-α</td>
<td>CCCAGGCAGTCAGATCATCTTC</td>
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<td>GTAGTGGTGGGGGAGATTCG</td>
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<td>IL-6</td>
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<td>GTGCCCTTTGCTGCTTTCAC</td>
<td>81</td>
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</tr>
<tr>
<td>β-actin</td>
<td>CCCAGGCGGAGCGGAAGAT</td>
<td>GTCCGCGGAGGCCAGTCG</td>
<td>219</td>
<td>Zhi et al., 2007</td>
</tr>
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Effects of diosgenin on gene expression of proinflammatory cytokines in macrophages induced by *N. fowleri* lysate

We evaluated whether diosgenin regulates cytokine release from macrophages at the level of transcription. The gene expression of proinflammatory cytokines in untreated and diosgenin-treated macrophages induced by *N. fowleri* lysate was measured by quantitative real-time RT-PCR.

*N. fowleri* lysate and LPS significantly increased TNF-α gene expression in untreated macrophages over time, reaching 24.63-fold and 11.74-fold increases, respectively, compared with untreated macrophages at 12 h post-incubation. In contrast, TNF-α gene expression in diosgenin-treated...
Fig. 2- Morphological characteristics of (A) untreated human monocyte U937-derived macrophages, (B) diosgenin-treated macrophages, (C) untreated macrophages induced by *N. fowleri* lysate, (D) diosgenin-treated macrophages induced by *N. fowleri* lysate (CC_{50}), (E) untreated macrophages induced by LPS, and (F) diosgenin-treated macrophages induced by LPS (5 µg/ml). Cells were observed under an inverted microscope for 72 h at 400× magnification.
Fig. 3 - Real-time quantitative RT-PCR analysis. Gene expression of proinflammatory cytokines (A) TNF-α, (B) IL-1β, and (C) IL-6 in untreated and diosgenin-treated macrophages induced by LPS or *N. fowleri* lysate was measured using β-actin as a control. Data represent the means ± SE of three independent experiments. Statistical analysis was performed by comparison with LPS or *N. fowleri* lysate. *p < 0.05.*
macrophages induced by *N. fowleri* lysate or LPS was not found at the indicated times (Figures 3A and 4). Untreated and diosgenin-treated macrophages induced by *N. fowleri* lysate or LPS showed significant increases in both IL-1β and IL-6 gene expression, although the gene expression levels of IL-1β were different from those of IL-6 at the indicated times (Figures 3B, C and 4). Our data revealed that diosgenin had proinflammatory effects via TNF-α, but not IL-1β or IL-6.

**DISCUSSION**

The present study evaluated the cytotoxicity of diosgenin in human monocyte U937-derived macrophages by MTT assays. Our study demonstrated that the MNTD of diosgenin in macrophages was 100 mg/ml at 24, 48, 72 h, which was different from our previous study using human neuroblastoma SK-NMC cells (250 mg/ml) (Rabablet et al., 2015). This result suggests that diosgenin has higher cytotoxicity in human macrophages than in SK-NMC cells. Furthermore, the cytotoxicity of diosgenin in macrophages was dose dependent (Figure 1A), whereas in SK-NMC cells, it was dose and time dependent (Rabablet et al., 2015). The cytotoxicity of *N. fowleri* lysate in macrophages was also determined by MTT assays. The CC50 of *N. fowleri* lysate in macrophages was 8 mg/ml (Figure 1B), whereas that in rat microglia is 1 mg/ml (Lee et al., 2011), suggesting that *N. fowleri* lysate had less cytotoxicity in human macrophages than in rat microglia.

*N. fowleri* lysate and LPS induced morphological changes in untreated macrophages, such as disruption and breakdown of the plasma membrane and a large rounded shape (Figure 2C, E). However, *N. fowleri* lysate and LPS induced slightly different morphological changes in macrophages treated with diosgenin at the MNTD (Figure 2D, F), suggesting that diosgenin can suppress the effects of *N. fowleri* lysate and LPS in macrophages.

The gene expression of proinflammatory cytokines TNF-α, IL-1β, and IL-6 in macrophages induced by *N. fowleri* lysate was measured by quantitative real-time RT-PCR. We found upregulation of these genes over time after stimulation by *N. fowleri* lysate, although the degree of changes was different (Figures 3 and 4). Similar expression patterns of these genes were observed after stimulation with LPS. Our data were consistent with a report by Chokri et al., (1989), revealing that activated macrophages from C57BL/6 mice produce TNF-α, IL-1β, and IL-6 after stimulation with LPS. Jung et al., (2010) proposed that diosgenin inhibits production of IL-1β and IL-6.

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**Fig. 4-** Real-time PCR analysis and agarose gel electrophoresis. Gene expression of proinflammatory cytokines TNF-α, IL-1β, and IL-6 and the housekeeping gene β-actin was measured in LPS- and *N. fowleri* lysate-induced macrophages in the absence or presence of diosgenin. Data represent the means ± SE of three independent experiments. Statistical analysis was performed by comparison with LPS or *N. fowleri* lysate. * p < 0.05.
6, but not TNF-α, in interferon-γ-stimulated mouse Raw264.7 macrophages induced by LPS. In contrast, our study showed that diosgenin inhibited gene expression of TNF-α, but not IL-1β or IL-6 (Figure 3A). This result suggested that diosgenin inhibited the production of inflammatory mediators induced by LPS/N. fowleri lysate in human monocyte U937-derived macrophages. This action results from suppression of TNF-α at the transcriptional level.

In conclusion, our data showed that human monocyte U937-derived macrophages can be used as a model to study the gene expression of proinflammatory cytokines that are believed to be important in the pathogenesis of PAM. Additionally, this cell type can be used to study the effect of diosgenin on proinflammatory cytokines.

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