INTRODUCTION

Innate immunity is one of the major responses against pathogenic infections by bacteria, virus, and fungi. Macrophages are the cells representative of innate immune functions and are therefore regarded as important cells in managing anticancer, antibacterial, antifungal, and antiviral responses [1-3]. Macrophages are a fully differentiated form of monocyte derived from hematopoietic stem cells. Major functions carried out by these cells include phagocytic uptake of infective pathogens, the release of toxic radical molecules such as reactive oxygen species (ROS), the stimulation of T cells by antigen presentation and by producing costimulatory signals via CD80 and CD86 antibodies, but not by cell-extracellular matrix (fibronectin) adhesion. Similarly, the surface levels of CD29 and CD43 were increased by 2H-PPD exposure. Therefore, our results strongly suggest that 2H-PPD has the pharmacological capability to upregulate the functional role of macrophages/monocytes in innate immunity.

Keywords: Panax ginseng, 20S-dihydroprotopanaxadiol, Phagocytosis, Reactive oxygen species generation, Adhesion
[9-11]. Owing to the improvement of numerous chemical and biological techniques, different types of ginseng-derived metabolites or derivatives such as compound K, ginsenoside F1, and 20S-dihydroprotopanaxadiol (2H-PPD) have been identified and developed as herbal remedies [12-14].

Thus far, no papers have reported the pharmacological action of 2H-PPD (Fig. 1A), a protopanaxadiol (PPD) derivative. Because its source compound, PPD, is known to exhibit various biological activities, including anti-cancer, pro-apototic, and anti-inflammatory effects [15-17], exploring the pharmacological actions of 2H-PPD could be beneficial for developing novel ginseng-derived compounds. Therefore, in this study, we investigated the immunomodulatory role of 2H-PPD on the innate immune responses of macrophages and monocytes.

MATERIALS AND METHODS

Materials

2H-PPD was obtained from Ambo Institute (Daejeon, Korea). The purity of the compound was greater than 98% according to HPLC analysis. Lipopolysaccharide (LPS) and sodium nitroprusside (SNP) were obtained from Sigma (St. Louis, MO, USA). RAW264.7 and U937 cells were purchased from American Type Culture Collection (Manassas, VA, USA). Cell–cell-adhesion-inducing antibodies to CD43 and CD29 were used as reported previously [18,19]. Antibodies to CD82 and CD86 were purchased from BD Bioscience (San Diego, CA, USA).

Cell culture

RAW264.7 and U937 cells were cultured in RPMI1640 with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin at 37°C in a humidified 5% CO₂ atmosphere.

Cell viability assay

Cell viability and the extent of proliferation were assessed by a conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolum bromide (MTT) assay [4]. RAW264.7 and U937 cells (5×10⁴ cells/well) were incubated with various concentrations of 2H-PPD (0 to 60 µM) for 24 h and were further incubated with MTT solution (0.5 mg/mL) for an additional 4 h at 37°C. The absorbance of the samples was measured at 490 nm using a microplate reader (Molecular Devices, Menlo Park, CA, USA).

Determination of phagocytotic uptake

To measure the phagocytic activity of RAW264.7 cells, we modified a method reported previously [20]. RAW264.7 (5×10⁴) cells treated with 2H-PPD (0 to 40 µM) were resuspended in 100 µL PBS containing 1% human AB serum and incubated with fluorescein isothiocyanate (FITC)-dextran (1 mg/mL) at 37°C for 2 h. The incubations were stopped by adding 2 mL ice-cold phosphate-buffered saline (PBS) containing 1% human serum and 0.02% sodium azide. The cells were then washed three times with cold PBS-azide and analyzed on a FACScan flow cytometer, as reported previously [21].

Determination of reactive oxygen species generation

The level of intracellular ROS was determined by a change in fluorescence resulting from the oxidation of the fluorescent probe, dihydrorhodamine 123 (DHR123). Briefly, 5×10⁵ RAW264.7 cells were exposed to 2H-PPD (0 to 60 µM) for 30 min. After incubation, cells were then incubated with SNP (0.25 mM), an inducer of ROS production, at 37°C for 2 h. Cells were incubated with 20 µM of the fluorescent probe DHR123 for 1 h at 37°C. The degree of fluorescence, corresponding to intracellular ROS, was determined using a FACScan flow cytometer (Beckton-Dikinson, San Jose, CA, USA), as reported previously [21].

Flow cytometric analysis

The expression of co-stimulatory molecules (CD80 [16-10A1, 1:50] and CD86 [GL1, 1:50]) in RAW264.7 cells was determined by flow cytometric analysis [4,5]. The RAW264.7 cells (2×10⁶ cells/mL) treated with 2H-PPD (0 to 40 µM) for 12 h were washed with a staining buffer (containing 2% rabbit serum and 1% sodium azide in PBS) and incubated with directly labeled antibodies for a further 45 min on ice. After washing three times with staining buffer, stained cells were analyzed on a FACScan flow cytometer (Becton-Dickinson).

Cell-cell or cell-extracellular matrix protein (fibronectin) adhesion assay

A U937 cell-cell adhesion assay was performed as reported previously [4,22]. Briefly, U937 cells were pre-incubated with 2H-PPD (0 to 40 µM) for 1 h at 37°C, and they were further incubated with function-activating (agonistic) anti-CD29 or anti-CD43 antibodies (1 µg/mL each) in a 96-well plate. After a 30 min incubation, cell-cell clusters were identified by a homotypic cell-cell adhesion assay using a hemocytometer [4,5] and photo-
tographed with an inverted light microscope equipped with a COHU high-performance CCD (Diavert) video camera. For a cell-fibronectin adhesion assay, U937 cells (5×10^5 cells/well) were seeded on a fibronectin (50 μg/mL)-coated plate and incubated for 3 h [23]. After removing unbound cells with PBS, the attached cells were treated with 0.1% of crystal violet for 15 min. The optical density value at 570 nm was measured by a Spectramax 250 microplate reader.

**Statistical analysis**

Student’s t-test and one-way ANOVA were used to determine the statistical significance of the differences between values for the various experimental and control groups. Data are expressed as mean±SEM, and the results were obtained from at least three independent experiments performed in triplicate. A p-value of 0.05 or less was considered statistically significant.

**RESULTS AND DISCUSSION**

Numerous studies have reported that ginseng and its ingredients can modulate immune functions [10,24]. However, the specific compounds from ginseng carry out regulation of various immunological responses are not fully understood. Here, we aimed to develop a synthesizable compound from ginseng-derived components with immunomodulatory properties. 2H-PPD is a representative derivative of PPD, which can now be produced by synthetic methods [8]. Therefore, we investigated the regulatory function of 2H-PPD (Fig. 1A) on the innate immune responses of macrophages and monocytes, such as phagocytic uptake, ROS generation, costimulatory molecule expression, cell-cell adhesion, and regulation of surface adhesion molecules in monocytic and macrophage-like cells such as RAW 264.7 murine macrophages and U937 human monocyte cells, respectively. Interestingly, without altering the viability of RAW264.7 and U937 cells (Fig. 1B), 2H-PPD significantly enhanced the phagocytic uptake of FITC-dextran in RAW264.7 cells (Fig. 2), indicating that this compound could stimulate innate immune responses by macrophage cells, including engulfment of bacterial, virus, or fungi. Moreover, this compound enhanced the release of SNP-derived radicals (Fig. 3), suggesting a putative role of indirect antibacterial activity via toxic molecule generation, as has been reported with other radical generating compounds [25]. In addition, 2H-PPD also stimulated an increase in surface levels of costimulatory molecules such as CD80 (Fig. 4A) and CD86 (Fig. 4B), essential molecules giving...
ing signal 2 for boosting the interactions of MHC class II/T cell receptor [26] in RAW264.7 cells. Although LPS displayed higher induction activity of costimulatory molecules (Fig. 4C) as reported previously [26,27], these results indicate that macrophages can be functionally stimulated by 2H-PPD treatment.

To remove infected pathogens, monocytes/macrophages must migrate into inflamed tissue. For this process, the functional activation of adhesion molecules is essential [28]. Representative adhesion molecules of macrophages/monocytes are CD29, CD98, CD147, and CD43 [4,5]. Therefore, whether 2H-PPD can modulate the adhesion events mediated by CD29 and CD43 was examined using function-activating antibodies against CD29 and CD43. Interestingly, this compound boosted cluster formation between U937 cells (Fig. 5A, B), while it blocked the adhesion of U937 cell to the extracellular matrix protein fibronectin (Fig. 5C), which is mediated by β1-integrins [5]. Furthermore, at 60 μM, 2H-PPD significantly increased surface levels of CD29 and CD43 (Fig. 5D), suggesting that this compound has a predominant role in accelerating CD29- and CD43-mediated cell-cell interactions, but not cell-tissue interactions.

The innate immune functions of macrophages, such as phagocytosis, migration, and adhesion to other cells, require changes in membrane structures via actin cytoskeleton rearrangement [29,30]. As such, inhibition of changes in the actin cytoskeleton by actin cytoskeleton disruptors such as cytochalasin B has been found to diminish phagocytic uptakes, morphological changes, and cell adhesion [31,32]. Recently, we reported that ginsenoside Rp1, a ginsenoside derivative, is able to directly control the activation of the actin cytoskeleton regulatory protein, vasodilator-stimulated phosphoprotein [33]. Whether 2H-PPD is also able to regulate the actin cytoskeleton machinery in macrophages/monocytes has not yet been determined. Since phagocytosis and cell-cell adhesion are regulated by actin cytoskeleton changes [4,34,35], the mechanism by which 2H-PPD might control these events in terms of actin cytoskeleton rearrangement will be further evaluated in future studies.

In conclusion, we found that 2H-PPD can boost the
2H-PPD enhances monocyte and macrophage functions.

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Fig. 5. Effect of 20S-dihydroprotopanaxadiol (2H-PPD) on U937 cell adhesion. (A,B) U937 cells (1×10^6 cells/ml) pretreated with 2H-PPD were incubated in the presence or absence of pro-aggregative antibodies (1 μg/mL each) to CD43 (161-46) and CD29 (MEM-101A) for 30 min. Images of the cells in culture were obtained using an inverted phase contrast microscope attached to a video camera. Quantitative evaluation of U937 cell-cell clusters was performed by a quantitative cell-cell aggregation assay. (C) Effects of 2H-PPD on cell-fibronectin (FN) adhesion was examined with U937 cells pretreated with 2H-PPD by seeding on FN (50 μg/mL)-coated plates for 3 h. Attached cells were visualized by crystal violet assay, as described in Materials and Methods section. (D) The surface levels of CD29 and CD43 in U937 cells for 6 h were determined by flow cytometry. Data represent mean±SEM of three independent observations performed in triplicate. MFI, mean fluorescence intensity. *p<0.05 and **p<0.01 compared to control.

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