The Anti–Inflammatory Effects of *Picea wilsonii* Mast on HaCaT Cells

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HaCaT 세포주에서 *Picea wilsonii* Mast의 항염효과

차경재

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Atopic dermatitis refers to a chronic, recurrent, skin condition, typically typified by itching, inflamed skin. It precedes other allergic diseases, such as asthma, food allergies, and allergic rhinitis, and is usually accompanied by various other immune disorders and secondary symptoms. In this study, we discovered that when treating TNF-α and IFN-γ–stimulated HaCaT cells with various concentrations of *Picea wilsonii* Mast (PwM) extracts, the cell viability was excellent. In addition, we measured the inflammatory cytokines associated with atopic dermatitis, including IL-6, IL-8, IL-13, and MCP-1. The production of IL-6, IL-13, and MCP-1 decreased in the presence of PwM extracts, whereas there was no significant difference in the production of IL-8. Further studies are necessary to develop an effective cure for atopic dermatitis and inflammation using foreign plant extracts, and PwM efficacy should be determined with an in-depth, objective verification process using protein and mechanism analysis.

**Key words:** Atopic dermatitis, Keratinocytes, *Picea*, Tumor necrosis factor–alpha

**Introduction**

Atopic dermatitis (AD) is more common in Westernized and industrialized countries, where families are getting smaller; it is apparent in 10~20% of children and 1~3% of adults. This prevalence rate is similar in Korea, where the number of patients with AD has been soaring lately [1,2]. AD is a chronic, recurrent, and inflammatory disease that develops before other allergic illnesses such as asthma, food allergies, and allergic rhinitis, and often coexists with a number of other immune disorders and secondary symptoms [3,4]. At the onset of atopic dermatitis, scratching the skin damages the integrity of the skin barrier, worsening the inflammatory reaction. This is accompanied by erythema, lichenification, sores, and itchiness [5]. The disruption of the skin barrier makes it easier for antigens and stimulants to permeate the skin, resulting in increased production of inflammatory cytokines (IL-1, -2, -3, -4, -5, -6, -11, -13, TNF-α, and GM-CSF), and chemokines (IL-8, RANTES [regulated on activation, normal T cell expressed and secreted]), MIP-1α (macrophage inflammatory protein–1α), MCP-1 (monocyte chemotactic protein–1), MCP-3, MCP-4, and eotaxin. These...
cytokines and chemokines then interact with each other to produce atopic dermatitis, which is characterized by an increase in eosinophil, activation of mast cell, and abnormal generation of IgE (a reaction that leads to a vicious circle of inflammation). As a result, one of the potential mechanisms for AD treatment is the regulation of cytokine production [6-8]. *Picea wilsonii* Mast (PwM) trees grow up to 1.5 m in diameter and 50 m in height, and they have dark gray, rough bark that does not change with age. Their shape is that of a crown cone or cylinder, and their branches spread horizontally: new branches are initially yellowish-gray and free of hair, with sprout-less twigs as long as 6~8 mm. Once developed, the needles are dark green and 0.5~2 cm long; they have a diamond-like appearance with a flat top, and bend forward. The pollen cone is 20~30 mm long and pink, while the seed cone is 4~8 cm in length and changes from green (while young) to yellowish-brown (once ripened). The seed itself is 3~4.5 mm long, resembles an egg, and has wings of 9~11 mm; it is smooth and thin at the edge, but may also be hard and stiff. These PwM trees grow in north-central China from Qinghai and Sichuan to Nei Mongol, Shanxi, and Hubei, scattered among other spruces and hardwoods in upper montane forest (at 1,400~3,000 m) [9,10] (Fig. 1). In this study, we aimed to measure cell viability in TNF-α and IFN-γ-stimulated HaCaT (human adult low calcium high temperature) cells, and to observe whether *Picea wilsonii* Mast. PwM extracts inhibit cytokine secretion in cell culture media, thus identifying whether or not they could be a candidate treatment for AD. We also aimed to raise awareness of the need for research using foreign plant materials in the future.

### Materials and Methods

#### 1. Cell culture

Human keratinocyte HaCaT cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL). These cells were maintained at subconfluence in a 95% air, 5% CO2 humidified atmosphere, at 37°C.

#### 2. PwM preparation

PwM was purchased from the Foreign Plant Extract Bank (FPEB), and standard extracts have been deposited at the Herbarium of the department of Herbal pharmaceutical development (Korea Institute of Oriental Medicine, Daejeon, Korea) and the division of Life Science (Daejeon University, TUT, Daejeon, Korea) [11]. The whole plants of PwM (33.1 mg) were dried, powdered, and then extracted with dimethyl sulfoxide (DMSO; 0.3, 1, 3, 10, and 30 µg/mL) for 1 day at room temperature.

#### 3. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazollum bromide (MTT) assay

Cell viability was assayed based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by using a cell proliferation kit I (Roche Diagnostics, Mannheim, Germany). HaCaT cells in 100 µL of culture medium were seeded into a 96-well plate. PwM was added to the wells at a concentration ranging between 0.3 and 30 µg/mL. Following incubation for 24 h at 37°C, 10 µL MTT solution was added and the plates were incubated again for 4 h. Solubilization solution (100 µL) was subsequently added to the wells. Following 24 further hours of incubation, absorbance was measured at 550 nm using an ELx808

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**Fig. 1. Picea wilsonii Mast.** The tree grows up to 50 m tall, with a trunk up to 1.5 m in diameter. The bark is dark gray and rough, and remains scaly with age. It is found in north-central China, from Qinghai and Sichuan to Nei Mongol, Shanxi, and Hubei, where it is scattered among other spruces and hardwoods in the upper montane forest (1,400~3,000 m).
enzyme–linked immunosorbent assay (ELISA) reader (Bio–Tek Instruments Inc., Winooski, VT, USA).

4. Cell density determination

Cell numbers and viability were assessed by trypan blue (Sigma–Aldrich, MS, USA) dye exclusion. Twenty microliters of trypan blue solution were mixed with 10 μL of cell suspension in a microtube to obtain a final density of 1.5×10⁵ cells/mL, and these microtubes were loaded into a hemocytometer.

5. Enzyme–linked immunosorbent assay (ELISA)

Following pre–treatment with PwM for 30 minutes, HaCaT cells were treated with tumor necrosis factor–α (TNF–α) and interferon–γ (IFN–γ). The concentrations of IL–6, IL–8, IL–13, and MCP–1 in the supernatant were measured using sandwich ELISA, by using a BD OptEIATM sets (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s instructions. The cytokine concentration was calculated using a linear regression equation obtained from the standard absorbance values. All assays were performed in at least three independent experiments.

6. Statistical analysis

Values are expressed as the mean ± standard deviation. Data were analyzed with the Student’s t-test by using SPSS software, version 18.0 (SPSS Inc., Chicago, IL, USA). *p<0.05 and **p<0.01 were considered to indicate a statistically significant difference.

Results

1. Cytotoxic effects on HaCaT cells in vitro

Before studying the cytotoxic effects of PwM extracts, we determined the optimal dose of TNF–α and IFN–γ (10 μL). The PwM showed a cytotoxic effect on these cells: cell viability was altered after 24 h of PwM treatment using concentrations in the range of 0.3–30 μg/mL (Fig. 2). The results of negative controls and TNF–α and IFN–γ–stimulated positive controls revealed excellent cell viability of over 120%. This indicates that the cytotoxicity of PwM extracts is very low.

2. Effect of PwM extracts on cytokine levels in TNF–α and IFN–γ–stimulated HaCaT cells

Among the various cytokines and chemokines relating to acute or chronic atopic dermatitis, we observed the effects of PwM upon MCP–1, IL–6, IL–8, and IL–13 production in this study. In HaCaT cells, production of MCP–1 by the TNF–α and IFN–γ–stimulated positive control increased remarkably, compared to the negative control. When this positive control was subsequently treated with PwM extracts, MCP–1 production decreased significantly with increasing PwM concentrations, and this was particularly significant at a concentration of 3 μg/mL (p<0.01). The same was true for IL–6: the TNF–α and IFN–γ–stimulated positive control HaCaT cells showed a remarkable increase in IL–6 production, compared to the negative control. With PwM treatment under the same conditions, there was a significant reduction in IL–6 production with increasing PwM concentration (p<0.05). In the case of IL–8, a remarkable increase in IL–8 production occurred in the TNF–α and IFN–γ–stimulated positive control HaCaT cells, but there was no significant change with PwM treatment at the various concentrations.

In HaCaT cells, the production of TNF–α and IFN–γ–stimulated IL–13 rose noticeably compared to the negative control, with a remarkable decrease in production at PwM concentrations of 3–10 μg/mL or more (p<0.01). In conclusion, PwM extracts were found to inhibit the production of IL–6, IL–13, and MCP–1, and to cause no significant change in IL–8 production.
Discussion

The noticeable increase in cases of atopic dermatitis in countries that have been through rapid westernization and industrialization, like Korea, suggests that environmental factors have a strong effect on the development of atopic dermatitis. Some explain this with the "hygiene hypothesis," however this is insufficient explanation. Since it was reported in 2006 that function-loss mutations of filaggrin (FLG) were highly implicated in Caucasian patients with atopic dermatitis in north-west Europe, innate skin barrier damage has surfaced as the primary cause of atopic dermatitis [12]. Recently, views on the pathogenesis of atopic dermatitis have been divided into the outside-in model (that claims skin barrier damage is the primary cause), and the inside-out model (that holds that AD starts from abnormal immune reactions). Meanwhile, the outside-inside-outside model explains that these two mechanisms work together to cause AD [13]. In immunological terms, it is thought that atopic dermatitis is caused by various cells, such as T cells (particularly helper T cells), eosinophil and mast cells. T lymphocytes are involved in the formation of antibodies, and helper T (Th) cells infiltrate atopic dermatitis lesions. In terms of function, helper T cells are divided into Th1 cells and Th2 cells, and the latter are more prevalent in the acute stages of atopic dermatitis. Symptoms like itching, urticaria, erythema, blisters, and psoriasis appear in atopic dermatitis, as well as other dermatological immune reactions. Cytokines are an important ‘messenger’ protein secreted from cells. They are part of an extracellular signal delivery network that controls innate and adaptive immune reactions, and they are deeply involved in inflammatory reactions caused by viral infections and tissue damage. Therefore, observing changes in their secretion may facilitate an understanding of the development of this inflammatory and immunological reaction [14].

There are many Korean studies concerning the use of natural extracts in the treatment of atopic dermatitis. In particular, traditional medicinal extracts from Korean red ginseng, chestnut, and Chinese herbs [15], Scutellaria baicalensis water [16], Eucommia ulmoides [17], Opuntia humifusa [18], kimchi [19], and angelica [20] have been reported to mitigate the symptoms of atopic dermatitis. Despite extensive research, however, an innovative cure for AD has not yet been developed. With this in mind, we measured in vitro cell viability and inflammatory cytokine...
production in TNF-α and IFN-γ-stimulated HaCaT cells, with the aim of investigating the potential effect of PwM extracts on the treatment of atopic dermatitis. The HaCaT cells are made up of normal human epithelial keratinocytes. As shown in Fig 2, PwM extract has no cytotoxic effect on HaCaT cells were seeded into 96-well plate at 1.5 × 10^5 cells/well and were treated with PwM extract. In the course of inflammation, pro-inflammatory cytokines such as TNF-α, IL-1β, IL-4, IL-6, and IL-8 increase the number of activated inflammatory and immune-related cells, which cause the inflammatory state to spread. Eosinophil, monocyte, and lymphocyte migrate to the area and cause inflammation after chemokines of IL-8 and MCP-1 are released [21,22]. MCP-1 is one of the CC family chemokines, and it is an influential chemotactic factor for monocytes. MCP-1 is believed to regulate the migration and infiltration of monocytes, macrophages, memory T cells, and NK cells, and it has been implicated in various inflammatory disorders such as asthma and rheumatoid arthritis. Furthermore, in a study of patients with chronic and idiopathic urticaria, a chronic inflammatory skin disease, an increase in MCP-1 was observed. Finally, as MCP-1 increases the reactivity of monocytes and can easily facilitate the production of a pro-inflammatory environment [23–25]. IL-6 is an important inflammatory cytokine secreted from activated mast cells and a multi-functional protein that boosts acute phase protein synthesis in the innate immune response, thus contributing to the systemic inflammatory response [26]. IL-13 inhibits macrophage activation and controls inflammatory reactions such as atopic dermatitis: its dysregulation is known to be a major cause of allergic disorders, and it is generated excessively in the skin of patients with atopic dermatitis as the diseases develops [27]. Finally, IL-8 is a chemokine that guides neutrophils from blood vessels to tissues, and it is released by macrophages for the recruitment of other immune cells [28]. Thus the regulation of cytokine production such as IL-3, IL-4, IL-5, IL-6 caused by allergen is an important process in the pathogenesis and progression of allergic diseases. AD is characterized by elevated number of circulating eosinophils and their infiltration into the skin. Monocytes produce inflammatory cytokines and differentiate into inflammatory dendritic epidermal cells that induce the switch from Th2 to Th1/0 [29]. In the present study, inflammatory cytokine secretion was induced in HaCaT cell lines, and then PwM extracts were added at various concentrations. We found that these PwM extracts significantly inhibited the production of IL-6, IL-13, and MCP-1, but there was no significant change in IL-8 production. In Korea, most of the existing studies use indigenous anti-inflammatory plant extracts for the treatment of atopic dermatitis, but research using foreign plant materials has been insufficient. As far as the PwM extracts used in this study are concerned, objective and in-depth verification of their efficacy via protein and mechanism analysis is considered necessary. Furthermore, additional studies should follow using the various foreign plant extracts available, to determine their suitability for the treatment and alleviation of atopic dermatitis.

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References