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Research Paper

Effects of *Dictamnus dasycarpus* Turcz., root bark on ICAM-1 expression and chemokine productions in *vivo* and *vitro* study

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ABSTRACT

Ethnopharmacological relevance: The root bark of *Dictamnus dasycarpus* Turcz., family Rutaceae is a well known anti-inflammatory agent for skin diseases such as eczema, pruritus and urticaria in Eastern countries.

Materials and methods: We investigated the effects of methanol extract of *Dictamnus dasycarpus* root bark (MEDD) on Intercellular Adhesion Molecule-1 (ICAM-1) expression, epidermal hyperplasia and immune cell infiltration in 1-fluoro-2,4-dinitrofluorobenzene (DNFB)-induced contact dermatitis (CD) mice. We also investigated its effects on the expression of ICAM-1, binding capacity to THP-1 cells, cytokine and chemokine production, and phosphorylation of NF-κB in human keratinocytes (HaCaT cells).

Results: Topical application of MEDD effectively inhibited ICAM-1 expression and epidermal hyperplasia in inflamed tissues. MEDD treatment also inhibited immune cell infiltration induced by DNFB. In addition, treatment with MEDD reduced surface expression and total amount of ICAM-1in HaCaT cells and effectively lowered the capacity to bind to THP-1 cells. MEDD also lowered the levels of IL-6, IL-8, monokine induced by gamma interferon (MIG), monocyte chemotactic protein-1 (MCP-1) and regulated on activation, normal T cell expressed and secreted (RANTES). Finally, MEDD treatment prevented activation of the NF-κB pathway induced by TNF-α in HaCaT cells.

Conclusions: These data indicate that root bark of *Dictamnus dasycarpus* has the potential for treatment of inflammatory skin diseases as a complementary or alternative medicine to corticosteroids. In addition, they suggest that the anti-inflammatory effects of *Dictamnus dasycarpus* on CD are involved in the regulation of ICAM-1 expression and cytokine and chemokine secretion through down-regulation of the NF-κB signaling pathway in keratinocytes.

1. Introduction

Intercellular Adhesion Molecule-1 (ICAM-1), also known as Cluster of Differentiation 54 (CD54), is a member of the immunoglobulin superfamily, which is a superfamily of transmembrane proteins that includes antibodies and T-cell receptors. ICAM-1 binds to β_2 integrin lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18), which is found on all T cells, as well as on B cells, macrophages and neutrophils, and is involved in recruitment to the inflammatory site (Yang et al., 2005).

ICAM-1 is widely expressed on various cell types, including tissue macrophages and dendritic cells. ICAM-1 is also expressed on non-hematopoietic cells such as vascular endothelial cells, thymic and mucosal epithelial cells, and dermal fibroblasts (Long, 2011). It is also well known that ICAM-1 expression on keratinocytes precedes immune cell infiltration in contact dermatitis (CD) (Griffiths and Nickoloff, 1989).

The root bark of *Dictamnus dasycarpus* Turcz., family Rutaceae is a well known anti-inflammatory agent for skin diseases in

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Eastern countries. In traditional medicine, *Dictamnus dasycarpus* has primarily been used in treatments of skin diseases such as eczema, atopic dermatitis and psoriasis (Du et al., 2005). It can also kill worms, arrest itching, clear away heat and eliminate dampness (Kim et al., 1997).

Many bio-active components have been isolated from the root bark of *Dictamnus dasycarpus* such as prenylated flavanone, Limonoids, quinoline alkaloids, pyrrolidine alkaloids, sesquiterpenes and glycosides (Chang et al., 2001, 2002; Guo et al., 2012; Kang et al., 2000; Yang et al., 2011). *Dictamnus dasycarpus* and its isolated components have various properties including neuroprotective, immunosuppressive, anti-fungal and anti-allergic activities (Chang et al., 2002; Jeong et al., 2010; Jiang et al., 2008; Zhao et al., 1998). Recently, it has been reported that alkaloids including one new glycosidic quinoline alkaloid, 3-[1β-hydroxy-2-(β-D-glucopyranosyloxy)-ethyl)-4-methoxy-2(1H)-quinolinone, and fraxinellone, which is formed by the natural degradation of limonoids, have anti-inflammatory action (Kim et al., 2009; Yoon et al., 2012).

Our previous report demonstrated that *Dictamnus dasycarpus* exerted anti-inflammatory effects against CD induced by dinitrofluorobenzene (DNFB) in mice (Kim et al., 2013). In our previous studies, the methanol extract of *Dictamnus dasycarpus*, root bark (MEDD) effectively inhibited enlargement of ear swelling in CD mice. In addition, treatment with MEDD suppressed the increase in levels of IFN-γ and TNF-α, respectively. However, anti-inflammatory mechanisms related to chemokine production and ICAM-1 expression in keratinocytes are poorly understood.

Based on these backgrounds, we evaluated the effects of MEDD on ICAM-1 expression in inflamed tissues and chemokine production in keratinocytes. Specifically, the effects of MEDD on histopathological changes such as epidermal thickness, ICAM-1 expression and immune cell infiltration in ear tissues were assessed in *vivo*. The effects on surface expression and total level of ICAM-1, binding capacity to THP-1 cells, chemokine production and related mechanisms were investigated in *vitro*.

2. Materials and methods

2.1. Preparation of MEDD

The root bark of *Dictamnus dasycarpus* was purchased from Kwangmyungdang Medicinal Herbs (Ulsan, Korea). Fifty grams of *Dictamnus dasycarpus* root bark were immersed in 1000 ml of methanol and sonicated for 30 min, after which they were extracted for 24 h. The extract was then filtered through Whatman filter paper (No. 20) and evaporated under reduced pressure using a vacuum evaporator (Eyela, Japan). The condensed extract was subsequently lyophilized using a freeze dryer (Labconco, Kansas City, MO, USA). Finally, 2.8 g of lyophilized powder was obtained (yield, 5.6%). The methanol extract of *Dictamnus dasycarpus*, root bark (MEDD, Voucher no. MH2010-010) was deposited at the Division of Pharmacology, School of Korean Medicine, Pusan National University.

2.2. Animals

Male 6-week-old Balb/c mice were purchased from Samtaco (Incheon, Korea). Mice were housed under specific pathogen-free conditions with a 12 h light/dark cycle and free access to standard rodent food and water. All animal experiments were approved by our Animal Care and Use Committee and conducted according to institutional guidelines (PNU-2011-000406).

2.3. Induction of CD and experimental design

Mice were sensitized by painting 50 μL of DNFB (0.1%, v/v) in acetone:olive oil (AOO, 4:1) onto the shaved back of each mouse for 3 consecutive days. Four days after sensitization, each mouse was challenged by painting 30 μL of DNFB (0.2%, v/v) in AOO onto the dorsum of both ears every 2 days (4 times) (Kim et al., 2013). For the topical application of drugs, MEDD and DEX were dissolved in ethanol, filtered using a 0.45 μm pore size syringe filter and finally diluted in AOO (ethanol:AOO, 1:4). Thirty microliter of MEDD solution (30 or 300 μg/ear) was applied onto the dorsum of both ears every 2 days (4 times). In addition, naïve animals were treated with vehicles ($n=6$), while control animals (CTL) were sensitized and challenged with DNFB in AOO, then painted with vehicle ($n=8$). MEDD treated animals were sensitized and challenged with DNFB, then painted with 30 μg/ear of MEDD or 300 μg/ear of MEDD ($n=8$). Dexamethasone (DEX) treated animals were sensitized and challenged with DNFB, after which they were painted with 75 μg/ear of DEX. DEX was used as a positive control.

2.4. Evaluation of ICAM-1 immuno-expression and epithelial thickness

The formalin-fixed, paraffin-embedded tissue was cut using a microtome, mounted on slides, deparaffinised, and rehydrated. Antigen retrieval was conducted by boiling the slides for 15 min in citrate buffer (pH 6.0, Invitrogen) using a hot plate, then cooled for 25 min at room temperature (RT). After washing in phosphate buffered saline (PBS), endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 for 20 min at RT. Next, ICAM-1 antibody (1:100, Santa Cruz Biotechnology, Dallas, TX, USA) solution was applied overnight at 4 °C. The slides were subsequently washed in PBS and incubated with a SuperPicture™ 3rd Gen IHC Detection kit (Invitrogen, Grand Island, NY, USA). To detect the antigen–antibody complex, freshly prepared substrate 3,3′-diamonobenzidine tetrahydrochloride (DAB, Invitrogen) was applied onto the slides, which were counterstained with Mayer's hematoxylin (Sigma, St. Louis, MO, USA), mounted and examined under a light microscope (Olympus, Japan).

ICAM-1 immuno-expression was evaluated in a semi-quantitative manner. Briefly, we took photographs to select five random fields per slide using a light microscope ($\times 200$) with a digital camera. The ICAM-1 positive cells were grouped as follows: (a) 0 points, negative; (b) 1 point, 1–25%; (c) 2 points, 26–50%; (d) 3 points, 51–75%; and (e) 4 points, $>75\%$ (Qiu et al., 2014). The average ICAM-1 score of the control and each experimental group were represented as expression scores.

To measure the thickness of the epithelium, the vertical length between the basal lamina and the top of the outermost stratum granulosum was quantified. For each slide, 5 lengths were measured at random using Motic Images Plus 2.0 (Motic Instrument Inc., Hong Kong, China), and the average epithelial thickness of the control and each experimental group was used for analysis.

2.5. Evaluation of infiltrated immune cells

Tissue obtained from experimental animals was fixed in 10% neutral formalin overnight, then washed, dehydrated with ethyl alcohol sequentially, treated with xylene and embedded in paraffin. Sections were then stained with hematoxylin-eosin (H/E) and observed using a light microscope.

To evaluate the immune cell infiltration, the number of immune cells in connective tissue was counted in five photographs per slide ($\times 200$). Macrophages, polymorphonuclear leukocytes (PMNL), lymphocytes, eosinophils, plasma cells, and giant cells