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Immunosuppressive effects of fisetin against dinitrofluorobenzene-induced atopic dermatitis-like symptoms in NC/Nga mice

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ABSTRACT

Atopic dermatitis (AD) is a multifactorial chronic skin disorder that is increasing in prevalence globally. In NC/Nga mice, repetitive epicutaneous applications of 2-4-dinitrofluorobenzene (DNFB) induces AD-like clinical symptoms. Bioflanonol fisetin (3,7,3′,4′-tetrahydroxyflavone) is a dietary component found in plants, fruits and vegetables. Fisetin has various physiological effects that include anti-oxidation, anti-angiogenesis, anti-carcinogenesis and anti-inflammation. In this study, we investigated whether fisetin relieves AD-like clinical symptoms induced by repeated DNFB treatment in NC/Nga mice. Fisetin significantly inhibited infiltration of inflammatory cells including eosinophils, mast cells and $CD4^+$ T and $CD8^+$ T cells, and suppressed the expressions of cytokines and chemokines associated with dermal infiltrates in AD-like skin lesions. Total serum immunoglobulin E (IgE) levels and the ratio of phospho-NF-κB p65 to total NF-κB p65 were markedly reduced by fisetin. Fisetin also reduced the production of interferon-gamma and interleukin-4 by activated $CD4^+$ T cells in a dose-dependent manner, whereas the anti-inflammatory cytokine, interleukin-10 was increased. These results implicate fisetin as a potential therapeutic for AD.

1. Introduction

Atopic dermatitis (AD) is a chronically relapsing and pruritic inflammatory skin disease that clinically manifests as edema, erythematous, scaly and lichenified lesions that develop by cutaneous hyper-sensitivity, immunoglobulin E (IgE)-mediated sensitization and intense pruritus (Leung and Bieber, 2003). AD affects up to 18% of children and up to 5% of adults worldwide. The prevalence has increased approximately 3-fold over the past 30 years and its incidence is still increasing (Shaw et al., 2011). AD has a multiple and complex pathogenesis including exposure to chemical or environmental agents, and genetic or immunologic abnormality. This complexity has hindered the development of a truly efficacious AD treatment (Ong and Leung, 2006). AD is a biphasic inflammatory skin disease consisting of acute and chronic phases (Jin et al., 2009). In the acute phase, disruption of skin barrier function leads to invasion of external antigens including mites and microorganisms including bacteria, which induce a Th_2-dominant immune response (Nakagawa et al., 2011). In acute AD skin lesions, infiltrated Th_2 cells and the levels of associated cytokines such as interleukin (IL)-4, IL-5, and IL-13 are increased (Grewe et al., 1998). These Th_2-cytokines are associated with eosinophil infiltration, IgE hyper-production and mast cell activation. In contrast, the chronic phase of AD shows delayed-type hypersensitivity (DTH) and Th_1-dominant immune response by an interferon-gamma (IFN-γ) and IL-2 response that induces dermal thickening and tissue remodeling by excessive collagen accumulation (Hamid et al., 1994; Leung, 1999). In a recent study showed that the application of topical antigens and chemical haptens, such as 2,4,6-trinitrochlorobenzene (TNCB) or 2,4-dinitrofluorobenzene (DNFB), on murine skin induce AD-like clinical symptoms (Lee et al., 2007). DNFB is binds Lys and Cys residues in epidermal proteins, which evokes enhanced proliferation of effector T cells and disturbed epidermal barrier in murine models of AD (Saint-Mezard et al., 2004).

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NC/Nga mice showed clinical symptoms similar to those of AD in humans and so have been the most extensively studied animal model of AD (Vestergaard et al., 2000b). Under specific pathogen-free (SPF) conditions, the mice remain healthy, whereas in uncontrolled conventional conditions or upon the administration of a chemical hapten in SPF conditions, AD-like clinical symptoms with erythema, scaling, itching and dryness spontaneous develop at 6–7-weeks-of-age (Suto et al., 1999). Furthermore, serum IgE, thymus-and activation-regulated chemokine (TARC) levels are increased, which may play a critical role in recruiting Th_2 cells to the AD-like skin lesions (Vestergaard et al., 2000a). Fisetin (3,7,3′,4′-tetrahydroxyflavone) is commonly found in vegetables and fruits including strawberry, apple, persimmon, grapes and onion at concentrations ranging from 2 to 160 μg/g. Fisetin has broad pharmacological effects that include anti-oxidation, anti-inflammation, anti-carcinogenesis and anti-angiogenesis (Khan et al., 2013). Recent studies showed that fisetin suppresses cyclo-oxygenase (COX)-2 and prostaglandin E2 (PGE_2) expression in HT29 human colon cancer cells (Suh et al., 2009), inhibits histamine release and IL-4 expression in human mast cells (Park et al., 2008) and down-regulates the production of IL-4, IL-5 and IL-13 by activated basophiles in vitro (Higa et al., 2003). In vivo studies revealed that fisetin ameliorates lipopolysaccharide (LPS)-induced acute pulmonary inflammation (Geraets et al., 2009), streptozotocin-induced diabetes (Prasath and Subramanian, 2011) and ovalbumin-induced asthma. These reactions were related with the modulation of fisetin-mediated anti-inflammatory activity due to the inhibits activation of nuclear factor kappa-light chain enhancer of activated B cells (NF-κB) (Wu et al., 2011). Another recent study showed that fisetin attenuates Th_2-mediated allergic airway inflammation (Goh et al., 2012). However, the therapeutic efficacy of fisetin for AD is unknown. Since in most cases of AD patients have a familial history of allergies and bronchial asthma, the collective data to date support the suggestion that fisetin may be useful in the AD (Rajka, 1986). Presently, we investigated whether fisetin inhibits DNFB-induced AD-like clinical symptoms in a NC/Nga mice model.

2. Materials and methods

2.1. Animals

The six-week-old NC/Nga male mice (19–22 g) were purchased from Japan SLC (Shizuoka, Japan) and maintained under SPF conditions. Animals were housed in an air-conditioned animal room at 25 °C ± 1 °C at a relative humidity of 40% ± 5%, and were fed a laboratory diet and distilled water. Animal treatment and maintenance complied with the Principles of Laboratory Animal Care (NIH publication No. #85-23, revised in 1985) and with the guidelines issued by the ethical committee for animal welfare at Kyung Hee University [KHUASP (SE)-09-005]. All procedures were conducted in accordance with the United States National Institute of Health guidelines.

2.2. Drug treatment

Mice were randomly assigned to four treatment groups: DNFB non-treated group, DNFB treated group and DNFB with 20 mg/kg, and DNFB with 50 mg/kg, with five mice per group. Fisetin was dissolved in 20% ethanol with distilled water to a volume of 200 μl. DNFB non-treated group and DNFB group were treated with the same volume of 20% ethanol with distilled water. The fisetin-treated mice received oral fisetin at 20 or 50 mg/kg daily from days 8 to 15.

2.3. Allergen sensitization and challenge

DNFB sensitization were evoked by the repeated application of 25 μl of 0.15% DNFB in acetone/olive oil (3:1) to the outer and inner surfaces of the ears, and 100 μl of the same solution was applied to shaved dorsal skin once on days 3 and 7. On days 10 and 13, sensitized mice were challenged with 0.2% DNFB to the dorsal and ear skin surfaces. Control mice were treated with the same volume of vehicle. Increasing ear thicknesses were measured by a Digimatic Indicator thickness gauge (Mitsutoyo, Tokyo, Japan).

2.4. Dermatitis score

The severity of AD-like symptoms was macroscopically evaluated by the SCORAD method (Oranje et al., 2007). The degree of each symptom was graded from 0 to 3 (0, absence; 1, mild; 2, moderate; 3, severe). This scoring was based on the severity of edema, erythema, oozing, crust, excoriation, and lichenification. Overall dermatitis score was determined from the sum of all individual scores. Assessment was performed by an investigator who was blind to the grouping of the animals.

2.5. Measurement of cytokine production

T lymphocytes were isolated from the lymph nodes of mice, and $CD4^+$ T cells were purified using a Biomag separation column (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated $CD4^+$ T cells (1×10^6) were cultured in 24-well flat-bottom culture plates in RPMI-1640 medium with 10% heat-inactivated fetal bovine serum (FBS) and 50 μM β-mercaptoethanol, then stimulated with 5 μg/ml of immobilized anti-CD3 and 2 μg/ml of soluble anti-CD28 antibody for 50 h at 37 °C in a 5% CO_2 atmosphere. After incubation, culture supernatants were collected and the productions of IL-4, IL-10 and IFN-γ after T cell activation were quantified by ELISA (Biolegend, CA, USA).

2.6. Measurement of total and DNP-specific IgE

On day 16, total serum samples were prepared and quantified using OptEIA Set Mouse IgE ELISA kits (BD Pharmingen, San Diego, CA) and mouse anti-DNP IgE ELIAS kits (Alpha Diagnostic International, San Antonio, TX) according to the manufacturer's instructions. The absorbance was immediately read at 450 nm on an EL 800 ELISA reader (EL 800) (Bio-Tek, Winooski, VT).

2.7. RNA isolation and RT-PCR

Total RNA was extracted from excised dorsal skin using the Trizol method (Jeong et al., 2011). RNA (1 μg) was used as a template for cDNA synthesis. Reverse transcription was performed using 200 U/μL MMLV reverse transcriptase, 10 mM dNTPs, 5× reaction buffer, 0.02 μg/μL Oligo (dT) primers and 40 U/μL RNase inhibitor at 42 °C for 1 h. The reaction was stopped at 72 °C for 7 min, and cDNA products were stored at −20 °C. PCR was performed using the synthesized cDNA as a template and using specific primers for mice IL-5, IL-13, TARC, TNF-α and TSLP or β-actin, which was used as loading control. Primer sequences were as follows; IL-5, sense, 5′-ATGGAGATTCCCATGAGCAC-3′ and antisense, 5′-GTCTCTCCTCGCCACACTTC-3′, and IL-13, sense, 5′-CAGCATGGTATGGAGTGTGG-3′, and antisense, 5′-ACAGAGGCCATGCAATATCC-3′, and TARC, sense, 5′-TGCTTCTGGGGACTTTTCTG-3′, and antisense, 5′-CATCCCTGGAACACTCCACT-3′, and TNF-α, sense, 5′-CCGATGG GTTGTACCTTGTC-3′, and antisense, 5′-CGGACTCCGCAAAGTCTAAG-3′, and TSLP, sense, 5′-CGGATGGGGCTAACTTACAA-3′, and antisense, 5′-TCCTCGATTTGCTCGAACTT-3′, and β-actin, sense, 5′-CGGTTCCGATGCCCTGAGGCTCTT-3′, and antisense, 5′-CGTCACACTTCATGATGGAATTGA-3′. Amplified products were separated in 1% agarose gel and analyzed using a SLB Mylmager™ gel documentation system (UVP Inc., Upland, CA, USA).

2.8. Western blot analysis

For isolation of protein, the dorsal tissue was excised, and the fat was removed on ice, immediately placed in ice-cold lysis buffer. Lysates were centrifuged at 12,000g for 20 min, and protein concentrations were determined using the Bradford assay. Fifty micrograms of whole-cell lysate protein was separated electrophoretically on 10% Tris–glycine gels and subsequently transferred to PVDF membranes. For immunoblotting, membranes were blocked with 5% non-fat dried milk in Tris-buffered saline for 4 h at room temperature. Primary antibody against p-p65 (1:1000) was applied for 16 h at 4 °C or antibody against p-65 (1:1000) was applied for 6 h at room temperature. After washing, horseradish peroxide-conjugated anti-rabbit IgG antibody was used as the secondary antibody to detect the aforementioned protein bands by enhanced chemiluminescence (WESTSAVEUPTM; Abfrontier, Seoul, Korea). Intensity quantified using a Labworks image acquisition and analysis software (UVP Inc., Upland, CA, USA).

2.9. Histological analysis

On day 16, right ears and dorsal skins were removed and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4). Fixed tissues were washed in sucrose solution and frozen in OCT compound. Cryostat sections (8 μm) were mounted on slides and dried overnight at room temperature, before being fixed in ice-cold acetone for 5 min. Slides were rehydrated in PBS and blocked with 5% normal goat serum in PBS containing 0.3% Tween 20 (washing buffer) for 1 h at room temperature. After washing, sections were incubated with primary anti-CD4 or anti-CD8 antibody overnight at room temperature then washed and incubated with Alexa-Fluor 488-conjugated goat anti-rat antibody for 4 h at room temperature. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Sections were mounted and images were captured using an Eclipse 50i