Inhibitory effect of galangin on atopic dermatitis–like skin lesions

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ABSTRACT

Galangin is a member of the flavonol class of flavonoids having anti-inflammatory and anti-oxidative potential. Previously we reported the inhibitory effect of galangin on the mast cell-mediated allergic inflammation. For incremental research, we investigated the effects of galangin on atopic dermatitis (AD)-like skin lesions and underlying mechanisms of action. We established an atopic dermatitis model in BALB/c mice by repeated local exposure of house dust mite (Dermatophagoides farinae) extract (DFE) and 2,4-dinitrochlorobenzene (DNCB) to the ears. Repeated alternative treatment of DFE/DNCB caused AD-like skin lesions. Topical application of galangin reduced AD symptoms based on ear thickness and histopathological analysis, in addition to serum IgE and IgG2a levels. Galangin inhibited mast cell infiltration into the ear and serum histamine level. Galangin suppressed DFE/DNCB-induced expression of interleukin (IL)-4, IL-5, IL-13, IL-31, IL-32, and interferon (IFN)-γ in the ear tissue. To define the underlying mechanisms of action, tumor necrosis factor-α (TNF-α) and TNF-α-activated human keratinocytes (HaCat) model was used. Galangin significantly inhibited the expression of cytokines and chemokine by the down-regulation of transcription factor NF-κB and mitogen-activated protein kinases in HaCat cells. Taken together, the results demonstrate that galangin inhibited AD-like symptoms, suggesting that galangin might be a candidate for the treatment of AD.

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1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with a combination of intense pruritus, scratching, and cutaneous sensitization with allergens (Jin et al., 2009b). Pathophysiology of AD is complex and regulated by a multitude of genetic and environmental factors (Novak and Simon, 2011). Among environmental factors, house dust mite allergens are important for the development of AD, and Dermatophagoides farinae extract (DFE) is routinely used for the experimental induction of AD (Choi et al., 2013; Dai et al., 2011; Kwon et al., 2010). House dust mite allergens are known to contribute to pathogenesis of AD through the induction of Th2 cells to produce interleukin (IL)-4 and IL-13 (Guttman-Yassky et al., 2011b; Thomas et al., 2002). These cytokines induce immunoglobulin (Ig) E class switching and promote Th2 cell survival. Numbers of Th2 cells are elevated during the acute and chronic stages of AD. In addition, the cytokines they produce (IL-4 and IL-13) have direct effects on the epidermis, such as inducing keratinocytes to produce various proinflammatory cytokines and chemokines. These conditions are characterized by infiltrates of T cells, monocytes, macrophages, eosinophil and mast cells (Zimmermann et al., 2011). Mast cells have important roles in inflammation, and regulate eosinophil activation and recruitment (Guttman-Yassky et al., 2011a). Mast cell-derived histamine and inflammatory mediators contribute to itching and inflammation in AD (Oyoshi et al., 2009).

The pharmacological control of AD is an important clinical issue. A large number of studies have dealt with the effect of topical or systemic administration of corticosteroids, anti-histamines and calcineurin inhibitors (Quemeneur et al., 2003). However, it is well known that prolonged use of glucocorticoids and immune suppressors causes a variety of side effects. Recently, several effective therapies and agents involving natural materials have been introduced for AD treatment. Galangin (3,5,7-trihydroxyflavone) is a member of the flavonol class of flavonoids, and is present at high concentration in propolis, a natural material produced in honey and Alpinis officinarum, a plant which is a common herbal medicine in Asia (Heo et al., 2001). Galangin possesses certain pharmacological activities, including anti-mutagenic, anti-oxidative, and radical scavenging (Cushnie and Lamb, 2006; Gwak et al., 2011; Heo et al., 2001). We previously reported that galangin inhibited mast cell-derived allergic inflammation (Kim et al., 2013). Galangin also...
showed anti-inflammatory effects on collagen-induced arthritis mice without toxicity via attenuation of RANKL-induced activation of JNK, p38 and NF-kB pathways (Huh et al., 2013). However, the role of galangin on AD has not been studied yet. The objective of this study was to elucidate the effects of galangin on the AD-like lesions and to define the underlying mechanisms of these effects.

2. Materials and methods

2.1. Animals

Six-week-old female BALB/c mice were purchased from SLC Inc. (Hamamatsu, Japan). The animals were housed with 5–10 mice per cage in a laminar air flow room maintained at a temperature of 22 ± 2°C with a relative humidity of 55±5% throughout the study. The care and treatment of the mice were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

2.2. Drugs and chemicals

Galangin was purchased from Sigma (St. Louis, MO). Dermatophagoides farinae extract (DFF, Greer Laboratories, Lenoir, NC) was used as an antigen. All other reagents were purchased from Sigma unless otherwise stated. DFF was dissolved in phosphate-buffered saline (PBS) containing 0.5% Tween 20, 2.4-Dinitrochlorobenzene (DNCB, 1%) was dissolved in acetone/olive oil 1:3 solution. DMEM, antibiotics, and trypsin-EDTA were obtained from Invitrogen (Grand Island, NY). Recombinant human TNF-α and IFN-γ were purchased from R&D systems (Minneapolis, MN).

2.3. Cell culture and viability

A human keratinocyte cell line, HaCaT, was maintained in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin G, 100 µg/ml streptomycin) at 37°C in 5% CO₂. Cell viability was determined using the 3-(4,5-dimethylthiazolyl)-2,5-diphenyl tetrazolium bromide assay (MTT, Sigma). After 24 h of treatment, MTI (5 mg/ml) was added into each well that contained a sample and incubated for 2 h. Isopropanol was added to dissolve the formazan crystals. Absorbance on each sample compared to that of the control, was calculated and expressed as percentage.

2.4. Induction of AD-like lesions in the mouse ear

The induction of AD-like lesions by DFF and DNCB was performed based on our previous research (Choi et al., 2013; Kwon et al., 2010). A schematic experimental procedure is described in Fig. 1A. Mice (n = 5) were divided into four groups, and the surfaces of both ear lobes were stripped five times with surgical tape (Nichiban, Tokyo, Japan). After stripping, 20 µL of DNCB (1%) was painted on each ear and then 20 µL of DFF (10 mg/ml) 4 days later. Treatment of DFF/DNCB was alternately applied to both ears once a week. Two weeks after the first induction, tail bleeding was performed to check the serum IgE level. After confirming an atopic condition by IgE level, ears were treated with galangin (1 or 5 mg/kg) by painting until the end of 4 weeks induction. Ear thickness was measured 24 h after DFF or DNCB application with a dial thickness gauge (Mitutoyo, Co., Tokyo, Japan).

On day 28, blood samples were collected by an orbital puncture. The plasma was stored at −70°C for further analysis. After blood collection, ears were removed and used for a histopathological analysis. Serum IgE and IgG2a levels were measured using an ELISA kit (BD Biosciences, Oxford, UK) according to the manufacturer’s instructions. Briefly, for the detection of total IgE or IgG2a, 96-well plates (Nunc, Wiesenbad, Germany) were first coated overnight with anti-mouse IgE or anti-mouse IgG2a antibody. After blocking of unspecific binding, detection was performed using biotinylated anti-mouse IgE or anti-mouse IgG2a antibodies and streptavidin conjugated HRP. The colorimetric reaction of peroxidase substrate (3,3’-diaminobenzidine, DAB) and the number of mast cells in five sites chosen at random was counted. Eosinophils were counted blinded in 10 high-power fields at a magnification of 400×. Dermal thickness was analyzed in H&E-stained sections viewed under a magnification of 100×. Thickness was measured in five randomly selected fields from each sample.

2.5. Histological observation

The ears were fixed with 10% formaldehyde and embedded in paraffin. Thin 5 µm sections were stained with hematoxylin and eosin. Infiltrated lymphocytes, thickening of the epidermis, and fibrosis in the dermis were observed by microscope. For measurement of mast cell infiltration, skin sections were stained with toluidine blue, and the number of mast cells in five sites chosen at random was counted. Eosinophils were counted blinded in 10 high-power fields at a magnification of 400×.
tendency of ear swelling of each group was similar until 2 weeks. After 3 weeks of AD-like skin lesion induction, galangin treated groups showed a significant improvement of ear thickness and photograph (Fig. 1B and C).

Microscopic analysis of the ear skin showed many alterations. These features, including hyperkeratosis, large ulcers, and acute inflammatory cell infiltration, were observed (Fig. 2A upper panel). Compared with AD-like skin lesions, galangin significantly decreased DFE/DNCB-induced epidermal and dermal thickness (Fig. 2B) and infiltration of eosinophils (Fig. 2C). Mast cells are important group of effector cells and a source of histamine in AD. Histamine is a representative symptom-inducing substance in AD (Guttman-Yassky et al., 2011a). Therefore, the infiltration of mast cells in AD site and the serum level of histamine were examined. Galangin inhibited the infiltration of mast cells (Fig. 2A lower panel and 2D) and serum histamine (Fig. 3A).

3.2. Effects of galangin on serum immunoglobulin and expression of cytokines

IgE production is associated with Th2 cellular response. On the contrary, IgG2a is associated with Th1 response (Bieber, 2010). To discriminate the role of galangin on the Th1 or Th2 response, we examined serum levels of IgE (total and DFE specific) and IgG2a. Compared with the AD, total IgE, DFE-specific IgE, and IgG2a were significantly reduced by the galangin (Fig. 3B–D).

The acute phase of AD is predominantly a Th2 response, while the chronic phase is characterized by Th1 response (Bieber, 2010; Oyoshi et al., 2009). We examined the effect of galangin on the expression of AD-related inflammatory cytokines from the ear tissue by the real-time PCR. All cytokines were upregulated in AD-like skin lesions, and galangin inhibited the expression of both Th1 (IFN-γ) and Th2 (IL-4, IL-5, IL-13, IL-31 and IL-32) cytokines (Fig. 4). These results suggest that galangin inhibits expression of both Th1 and Th2 cytokines in AD-like skin lesions. In addition, galangin plays a suppressive role in chronic skin inflammation elicited by AD-like skin lesions.

3.3. Effects of galangin on the activation of keratinocytes

After the observation of inhibitory effect of galangin on the AD models, a human keratinocyte model was used for clarifying the biological function and molecular mechanism of the galangin. Keratinocytes have a crucial role on immune responses during development of AD-like skin lesions. Keratinocytes produce inflammatory factors that promote chronic, self-amplifying loops of immune activation (Guttman-Yassky et al., 2011a). Therefore, TNF-α/IFN-γ-induced pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, and GM-CSF) were examined (Fig. 5).
and IL-6) and the chemokine CCL17 in keratinocytes are considered as the most important mediators for the development of AD (Vestergaard et al., 2000). HaCaT cells were pretreated with galangin for 1 h and then stimulated with TNF-α/IFN-γ for 6 h. The results of real-time PCR showed that galangin inhibited TNF-α/IFN-γ-induced gene expression of TNF-α, IL-1β, IL-6 and CCL17 in HaCaT cells (Fig. 5A). These results suggest that galangin mitigated the inflammatory response by the down-regulation of the pro-inflammatory cytokines and chemokine in AD-like skin lesions.

TNF-α/IFN-γ-induced pro-inflammatory cytokines and CCL17 are regulated by MAPKs and NF-κB (Kim et al., 2011). To confirm the role of MAPKs and NF-κB on the expression of TNF-α/IFN-γ-induced cytokine and chemokine, specific inhibitors for ERK (PD98059), p38 (SB203580), JNK (SP600125) and NF-κB (PDTC) were used. As shown in Fig. 5B, specific inhibitors for ERK, p38 and NF-κB significantly inhibited TNF-α/IFN-γ-induced CCL17 expression. However, inhibitor for JNK appeared not to inhibit CCL17 in HaCaT cells. Galangin did not show cytotoxicity up to 10 μg/mL in keratinocytes (Fig. 6A).

To investigate the mechanism responsible for the inhibitory effect of galangin on the cytokine expression, we investigated the effect of galangin on the TNF-α/IFN-γ-induced MAPKs and NF-κB. HaCaT cells were pretreated with galangin for 1 h, and then stimulated with TNF-α/IFN-γ for 20 min. As shown in Fig. 6B, treatment of TNF-α/IFN-γ showed activation of MAPKs and NF-κB. Galangin (10 μg/mL) decreased TNF-α/IFN-γ-induced phosphorylation of ERK and p38, and nuclear translocation of p65 NF-κB following degradation of IκBα. However, galangin did not reduce the activation of JNK. These results suggest that galangin might suppress TNF-α/IFN-γ-induced cytokines and chemokine by the reduction of ERK, p38 and NF-κB in keratinocytes.

4. Discussion

Propolis is a resinous mixture that honey bees collect from various plants. Several studies have demonstrated that propolis might act as potent anti-inflammatory agent against both acute and
chronic inflammation (Borrelli et al., 2002; Khayyal et al., 1993). Propolis is generally composed of flavonoids, phenolic acid, phenolic aldehydes, ketone, wax, and resin. Among them, chrysin and galangin have been identified as active components of propolis (Gomez-Caravaca et al., 2006). Based on the known anti-allergic and anti-inflammatory effects of propolis, we examined the role of galangin in AD-like skin lesions using in vivo and in vitro models.

Acute AD lesions exhibit spongiosis (epidermal intercellular edema), hyperkeratosis (thickening of the stratum corneum), and chronic lesions characterized by acanthosis (diffuse epidermal hyperplasia) and infiltration of lymphocytes and mast cells (Kawakami et al., 2009). In the present report, galangin mitigated typical and histological changes, such as severe ear thickness, ulcers, dermal and epidermal thickness, epidermal hyperplasia and infiltration of inflammatory cells (eosinophils and mast cells). Mast cells release a number of important signaling molecules, among which histamine has particularly potent pro-inflammatory activities (O’Mahony et al., 2011). Histamine mainly induces erythema and edema, and is likely to be a significant mediator in patients with AD (Kawakami et al., 2009). In addition, serum histamine levels were reported to be significantly higher in patients with AD than controls (Greaves, 2005; Herman and Vender, 2003). In our results, treatment with galangin reduced serum histamine and pathogenesis of the skin lesions in AD. According to our results, we speculate that galangin has inhibitory effect on the development of AD by the reduction of histamine following the decrease of edema.

In AD patients, elevated total IgE and specific IgE to environmental allergens can be detected (Schmid-Grendelmeier et al., 2001). Langerhans cells of AD skin bearing the high-affinity IgE receptor FcεRI on their surface provide a link between environmental allergen exposure and Th2 cell activation (Dokmeci and Herrick, 2008). Th2 cell activation evokes expression of IL-4, IL-5, and IL-13 which regulating IgE class-switching by B cells (Dokmeci and Herrick, 2008). Levels of IL-31 also correlate with levels of Th2 cells in the skin of subjects with AD (Neis et al., 2006). IL-31 transgenic mice developed spontaneous pruritus and hallmarks of AD skin lesions (Brandt and Sivaprasad, 2011). In addition, it is known that IFN-γ drives the production of IgG2a (Jin et al., 2009a). In our AD model, galangin markedly inhibited the expression of Th1 (IFN-γ) and Th2 (IL-4, IL-5, IL-13, and IL-31) cytokines, and serum level of IgE and IgG2a. These results imply that galangin suppressed both Th1 and Th2 responses. Recently, it has been reported that IL-32 is expressed in lesionsal skin of AD patients, and contributes to the pathophysiology of AD (Meyer et al., 2010). In our results, galangin markedly inhibited DFE/DNCB-induced Th1 and Th2 cytokines and IL-32 in ear tissue. These results imply that galangin reduced both acute and chronic AD symptoms. Furthermore, our data suggest that IL-32 is a target of galangin in AD.

Keratinocytes produce cytokines and chemokines which are involved in the development of inflammatory skin disorders via several mechanisms, including the induction of Th17 cells. In AD mouse models, galangin was found to inhibit the expression of IL-17, IL-22, and IL-23, which are known to be key mediators of Th17 responses. In addition, galangin downregulated the expression of RANTES (CCL5), MCP-1 (CCL2), and IL-8, which are involved in the recruitment of inflammatory cells to the skin.

In summary, galangin is a promising candidate for the treatment of AD. It inhibits the expression of key inflammatory cytokines and chemokines, and reduces the levels of histamine and IgE. Further studies are needed to elucidate the mechanisms by which galangin exerts its anti-inflammatory effects in AD.
especially AD (Kwon et al., 2012). TNF-α and IFN-γ synergistically induce cytokines for AD symptoms in keratinocytes, and this experimental model has been widely used for imitating AD symptoms in vitro (Leung and Bieber, 2003; Leung et al., 2004; Vestergaard et al., 2000). In our results, galangin suppressed TNF-α/IFN-γ-induced CCL17, IL-6, and TNF-α and IL-1β. Recently, we demonstrated that galangin inhibits gene expression of TNF-α, IL-1β, and IL-6 in mast cells (Kim et al., 2013). These accumulated reports support our results showing the diminution of pro-inflammatory cytokines in keratinocytes. CCL17 and cytokine production in keratinocytes is upregulated by TNF-α/IFN-γ through the NF-κB pathway (Kwon et al., 2012; Saeki and Tamaki, 2006). Galangin inhibited the degradation of IκBα and nuclear translocation of NF-κB.

The MAPK cascade is also an important signaling pathway in immune responses and cross talks with NF-κB to regulate inflammation signaling (Arbabi and Maier, 2002). Previous studies reported that TNF-α/IFN-γ-stimulated keratinocytes showed activation of three types of MAPKs; ERK, JNK, and p38 (Kim et al., 2011). It has been reported that p38, but neither ERK nor JNK is involved in TNF-α/IFN-γ-stimulated expression of CCL17 in HaCaT cells (Nakayama et al., 2004; Qi et al., 2011). However, we observed that galangin significantly inhibited the activation of ERK and p38 in HaCaT cells. This study provides evidence that the inhibitory effect of galangin on the expression of pro-inflammatory cytokines and CCL17 is mediated by blocking ERK, p38 MAPKs and NF-κB pathways in HaCaT cells.

In conclusion, the present study demonstrates that topical application of galangin suppresses the development of AD-like skin lesions in vivo and in vitro models. Galangin inhibits the important cytokines for AD by the reduction of NF-κB and MAPKs (ERK, p38) in HaCaT cells. We provide evidence that galangin could be a potential therapeutic candidate for AD. Our data imply that galangin may be natural remedies and effectively prevent atopic dermatitis, and can be used as a useful pharmacological agent or food supplement.
Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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