Anti-Inflammatory Effect of Mallotus Philippinensis Bark Extracts in a Mouse Atopic Dermatitis Model

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Introduction

The Muell-Arg (Euphorbiaceae) plant Mallotus philippinensis is widely distributed and is used in traditional medicine [1]. Various parts of the plant are used for treating diabetes, and in wound healing. Recently, our group demonstrated that ethanol extracts of Mallotus Philippinensis Bark (EMPB) promoted migration of mesenchymal stem cells (MSCs), enhanced MSCs homing to the wound sites, and improved remodeling in the wound healing process in a mouse model [2]. MSCs are considered to be important during the early inflammatory phase of wound healing [3], and MSCs release cytokines that activate skin cells [4]. The inflammatory response causes recruitment and activation of many cell types and is closely regulated for the subsequent stages of proliferation to be initiated. However, little is known about the direct effect of EMPB on inflammatory response in vitro and in vivo.

Atopic dermatitis (AD) is a very common, chronic inflammatory skin disease in industrialized countries [5]. Dry skin, erythema, edema, lichenification, excoriations, oozing and crust are key clinical manifestations of AD. AD pathogenesis has not been clearly elucidated, though skin barrier defects and altered immune responses are key components in disease development [6]. The AD onset and extension are highly influenced by genetic and environmental factors. The necessary effect of treatment is primarily reductions in skin inflammation, while minimizing side effects. Therefore, the development of natural products with multipotent anti-inflammatory action to effectively treat AD is important. In this study, we evaluated whether EMPB has an effective action on anti-inflammation using an in vitro and in vivo model.

Materials and Methods

Preparation of the extract

Mallotus philippinensis from Nepal was purchased from Maruzen Pharmaceuticals Co Ltd (Hiroshima, Japan). Chopped bark from Mallotus philippinensis was extracted for 2 h with aqueous ethanol (80%, v/v) by reflux extraction. The ethanol concentration of the isolated extract was adjusted 50% v/v, then the extract was dried and collected as an EMPB powder.

Mice

In vivo experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Charles River Laboratories Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of Charles River Laboratories Japan.
Induction of dermatitis

Ten-week-old female NC/NgaTndCrj mice were housed in a controlled room with a 12:12-hour light-dark cycle and free access to laboratory chow and water. Mice were randomly divided into the following two groups (n=30 for each group) at the time of initiation: vaseline-treated group (vehicle), and EMPB in vaseline-treated group (EMPB). AD-like skin lesions were induced in NC/Nga mice by topical application of Biostir-AD (Biostir, Kobe, Japan), a cream-containing extract of house dust mite (Dermatophagoides farinae) [7]. The hair on the upper dorsal skin and the back of the ears of mice was shaved, then 4% (w/v) Sodium Dodecyl Sulfate (SDS) was applied to the shaved dorsal skin and both surfaces of each ear, and Biostir-AD was applied to the skin area twice a week for a further 3 weeks. After 1 week, 100 mg of vaseline (vehicle) or EMPB-vaseline (10 μg EMPB) was applied to the area once a day for a further 10 days. On day 30 of the experiment, mice were sacrificed, and the dorsal skin and ear were harvested for histological analyses. Any clinical signs related to toxicity, such as loss of body weight (BW), were evaluated in all animals during an experiment period.

Evaluation of the dermatitis score

The clinical features and dermatitis scores were evaluated using the criteria that have been already reported [8,9]. Briefly, the development of erythema, edema, erosion, dryness, scaling, and excoriation was scored as 0 (without symptoms), 1 (mild), 2 (moderate), and 3 (severe). The sum of an individual animal’s score was taken as the dermatitis score.

Cell culture

Murine macrophage RAW 264.7 cell line was used as an in vitro model to test the anti-inflammatory properties of EMPB. Cells were cultured in Eagle’s Minimum Essential Medium (MEM, NacalaiTeseq Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA) in a humidified atmosphere with 95% air and 5% CO₂ at 37°C. After being cultured in a 100 mm plastic dish and grown to 80% confluence, cells were detached and seeded into wells of a new plastic plate. After a 24 h recovery period, cells were further incubated for 24 h in MEM culture medium with or without EMPB at a concentration of 0.3 - 300 μg/mL in the presence or absence of 10 ng/mL lipopolysaccharide (LPS, NacalaiTeseq).}

Real-time PCR

Total RNA was extracted from RAW 264.7 cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instruction. cDNA was synthesized from 1 μg of RNA using QuantiTectRetrotranscriptase reaction (Qiagen). Real-time PCR (RT-PCR) was performed using SYBR green labeling (SYBR Premix Ex Taq II, Takara, Otsu, Japan) and a TP850 Real-Time PCR System (Takara), as previously described [10,11]. The primers used are listed in Table 1. GAPDH expression was used to normalize the samples, and each sample was run in triplicate. The relative fold change was then calculated based on the ΔΔCt method.

Histology

After sacrifice, the back skins and ears were fixed in 10% formalin neutral buffer solution for 24 h, embedded in paraffin, and thin-sectioned (4 μm thickness). The sections were obtained and stained with hematoxylin and eosine (H&E) to monitor histological changes in the skin. To measure infiltrated mast cells, sections were stained with toluidine blue, and the numbers of mast cells were counted.

Free radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent, which accepts an electron or hydrogen radical to become a stable molecule, is used to detect oxidant activities. The change in DPPH absorbance after the addition of different concentrations of EMPB and 0.1 mg/mL DPPH was determined to estimate the antioxidant capacity. Vitamin C (VC) was used as a positive control.

Statistics

The dermatitis score was analyzed using a Mann-Whitney U test. The results obtained in the cell culture study were analyzed using a two-tailed Student’s t-test. Data represented as mean ± Standard Error of the Mean (SEM) in all experiments. A p value < 0.05 was considered significant.

Results

EMPB treatment suppresses Biostir-AD-induced skin lesions in NC/Nga mice

The severity of dermatitis was evaluated for 30 days (Figure 1a). After the mite antigen was applied to the backs and ears of the mice, the clinical severity of the AD symptoms developed. Repeated application of Biostir-AD cream induced AD-like skin lesions in NC/Nga mouse. However, the EMPB treatment ameliorated the skin symptoms. The dermatitis score of back skin (Figure 1b), pinna skin (Figure 1c), and total score of both skins (Figure 1d) was determined by summating the severity index values of each mouse. In the EMPB treatment group, the dermatitis score was lower from day 21 (D21) to D30 than that in the vehicle group; especially, significance was observed at D27 (Figure 1b, 1c, 1d).

These results indicate that EMPB suppressed the spontaneously induced dermatitis in NC/Nga mouse (Figure 1e). During the experiment, changes in BW were measured to assess the general health status of mice (Figure 1f), and we confirmed that application of EMPB did not significantly affect the BW compared with the control group.
Figure 1: Effects of EPMB treatment on the development of atopic dermatitis-like skin lesions in NC/Nga mice. (a) The experimental scheme for induction of house dust mite-induced atopic dermatitis-like skin inflammation and EMPB treatment. Clinical severity scores of mice were measured on day (D) 20, 21, 24, 25, 27, 28, and 30 of the experimental period in the (b) back skin and (c) pinna skin. (d) Total score of both back skin and pinna skin. (e) House dust mite-induced skin lesions. Photographs were taken on the last day (D30) of the experimental period. (f) The changes in body weight (BW) of mice. Data represent mean ± SEM of all experiments. *p<0.05.
**EMPB improves Biostir-AD-induced histopathological features in NC/Nga mice**

Specimens from the back skin and ears of NC/Nga mice were examined histopathologically. As shown in Figure 2, H&E staining revealed AD-like symptoms, such as epidermal thickening (Figure 2a, b) and infiltration into the dermis (Figure 2c). However, EMPB treatment significantly reduced epidermal hyperplasia (Figure 2a, b) and immune cell infiltration (Figure 2c), suggesting that EMPB suppressed the Biostir-AD-induced inflammation. To investigate this phenomenon further, we measured the effect of EMPB on the infiltration of mast cells by staining with toluidine blue. In the EMPB treatment group, the number of the mast cells was decreased compared with the control group (Figure 2d, e).

**Effect of EMPB on expression of pro-inflammatory cytokines in macrophage cells**

In RAW264.7 macrophage cells, 10 ng/mL LPS stimulation increased the expression of pro-inflammatory cytokines such as interleukin (IL)-1β (Figure 3a). To observe the effect of EMPB on cytokine expression in RAW264.7 cells, the cells were treated with EMPB (0.3–300 μg/mL) in the presence of LPS (Figure 3b). EMPB reduced the IL-1β mRNA expression level in a dose-dependent manner from 0.3 to 300 μg/mL. Also, we determined the potential anti-inflammatory properties of EMPB by determining the mRNA expression levels of various kinds of inflammatory mediators at both 30 (Figure 3c) and 300 μg/mL (Figure 3d). Among pro-inflammatory mediators, EMPB treatment significantly reduced the expression of IL-1β, inducible nitric oxide synthases (iNOS), and cyclooxygenase (COX)-2 compared with the LPS-stimulated macrophage control cells. These results suggest that EMPB has beneficial effects in LPS-stimulated macrophages, supporting that EMPB may contain some useful anti-inflammatory components.

**Effect of EMPB on NF-κB activation and its free radical scavenging activity**

Nuclear Factor-Kappa B (NF-κB) is a master switch of inflammatory gene expression that regulates the levels of IL-1β and iNOS. Therefore, we investigated the effect of EMPB on the LPS-induced activation of NF-κB (Figure 4a). RT-PCR analysis showed that the mRNA expression levels of NF-κB component factors, such as p65, p50, and p52, were significantly suppressed in EMPB-treated cells compared with control cells.

AD patients were more prone to damage caused by reactive oxygen species (ROS) or oxidants [12]. Also, it has been shown that crosstalk between ROS levels and NF-κB signaling is involved in pathological conditions [13]. In this study, we confirmed that EMPB exhibited marked radical scavenging ability and scavenged 71.7± 12.1% of the DPPH free radical; the positive control (vitamin C) scavenged 91.8 ± 0.9 %. These results suggest that EMPB is effective for preventing AD by inhibiting the effect of inflammation, which is partly regulated by NF-κB activation and cellular ROS levels (Figure 4c).

**Discussion**

In the present study, we investigated the effects of EMPB on house dust mite-induced AD-like skin lesions in NC/Nga mice, an animal model of human AD [14,15]. Inflammatory responses play important roles in normal and pathological healing of diseases such as AD. Following pathologic onset, various factors activate the immune system, leading to a local inflammatory response [16]. Pro-inflammatory cytokine production, such as IL-6, IL-1β, and Tumor Necrosis Factor (TNF), and subsequent stimulation of oxygen-centered free radical production is induced. In addition, NF-κB constitutes a family of transcriptional regulator essential for inducible expression of many cellular signaling pathways, including iNOS, which is involved in cell survival and the inflammatory pathway [17]. Furthermore, inflammatory mediators such as IL-1β, and nitric oxide (NO) are produced by activated macrophages, which lead to fatal damage in inflammatory diseases. The high iNOS activity typically occurs in an oxidative environment, and high levels of NO induced by pro-inflammatory cytokines interact with superoxide anions leading to cell toxicity [18].

In this study, we used LPS-stimulated macrophages as an in vitro model of inflammation. The EMPB treatment attenuated LPS-induced inflammation. This study found that IL-1β, iNOS, COX-2, and NF-κB levels increased significantly in LPS-treated cells, whereas levels were decreased by treatment with EMPB. This supports that EMPB protects the cell and prevents inflammatory symptoms by decreasing the mRNA expressions of IL-1β, iNOS, COX-2, and NF-κB (Figure 4c).

In our preliminary screening experiment, EMPB was found to be activate MSCs migration and improved wound healing probably through anti-inflammatory action. MSCs may play an important role in tissue regeneration [19,20]. MSC also showed extensive immunomodulatory effects such as the secretion of various cytokines [20,21,22]. Previously, other group reported that EMPB components such as cinnamtannin B-1 exhibit antioxidant properties [23], antimicrobial activities [24], anti-platelet aggregation [25], and promotion of the migration of adipose tissue-derived stromal cells in vitro [26]. Therefore, the anti-inflammatory effect of EMPB in a mouse AD model may also be attributed to the induction of active MSCs.

In conclusion, we found that EMPB inhibited inflammatory responses induced by pro-inflammatory cytokines and NF-κB in LPS-stimulated macrophage cells. We also demonstrated that EMPB can ameliorate AD-like skin inflammation in a mouse model of AD in NC/Nga mice. These results may support the novel application of EMPB for treatment of AD and other inflammatory skin diseases.
Figure 2: Effects of EMPB treatment on histological features and infiltration of mast cells into the skins of NC/Nga mice. The sections from NC/Nga mice were stained with hematoxylin and eosin-stained dorsal skin lesions. (a, b) EMPB treatment reduced epidermal thickness. Data are representative of two independent experiments and presented as mean ± SEM of n=30 mice per group. (c) EMPB treatment reduced cellular infiltration into the dermis. (d, e) The number of mast cells stained by toluidine blue is reduced in the EMPB treatment group. Data represent mean ± SEM of all experiments. *p<0.05.
Table 1: Primer Sequences used in real-time PCR.

| Gene | Species | Forward Primer | Reverse Primer |
|------|---------|----------------|----------------|
| GAPDH | mouse   | AGCTTGTCATCAAGGGGAAG | TTGTAGTTAAGGGTTCGG |
| IL-1ß | mouse   | CAGGAGCAATGATGAGAAGA | TGTGCTATCCCTGGAAAGGTC |
| iNOS | mouse   | GTGGTGACAAAGCAATTGCTG | AAGGCCAAACACAGCATACCA |
| COX-2 | mouse   | AGAAGGAATGCTGACAGAA | CCCTAAAGATAGCATCTGGA |
| p65  | mouse   | TCAATGGCTACACAGAGCA | GGCAGAGGTCAGCTCATAG |
| p50  | mouse   | CACCTAGCTGCAAGAGG | GCAGGCTATTGCTCATACCA |
| p52  | mouse   | TCAATGGCAGAGTTCCACG | CCACTCCCTTCTCTGCTTG |

Figure 3: Effects of EMPB on pro-inflammatory mediators in the LPS-stimulated RAW264.7 cells. (a) LPS stimulation increased IL-1ß mRNA expression levels in RAW264.7 macrophage cells. (b) Cells were treated with various concentrations of EMPB ranging from 0.3 to 300 μg/mL in the presence of LPS. Dexamethasone (DEX) is the positive control of anti-inflammatory effect assay with a concentration of 0.1 μM. RT-PCR analysis showing mRNA expression of pro-inflammatory mediators in the LPS-stimulated cells with (c) 30 or (d) 300 μg/mL EMPB. Data represent mean ± SEM of all experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure 4: Effects of EMPB on the LPS-induced activation of NF-κB and ROS levels. (a) The mRNA expression levels of p65, p50, and p52 were significantly inhibited by the treatment of 300 μg/mL EMPB in the LPS-stimulated RAW264.7 cells. (b) The effect of anti-oxidative activity assays on EMPB at different concentrations. Vitamin C (VC) is the positive control of DPPH radical scavenging capacity assay. Data represent mean ± SEM of all experiments. **p<0.01, ***p<0.001, ****p<0.0001. (c) Schematic illustration of anti-inflammation mechanisms of EMPB.
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