ORIGINAL

Treatment with DHA/EPA ameliorates atopic dermatitis-like skin disease by blocking LTB4 production

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Abstract : Atopic dermatitis (AD) is caused by both dysregulated immune responses and an impaired skin barrier. Although leukotriene B4 (LTB4) is involved in tissue inflammation that occurs in several disorders, including AD, therapeutic strategies based on LTB4 inhibition have not been explored. Here we demonstrate that progression of an AD-like skin disease in NC/Nga mice is inhibited when docosahexaenoic acid (DHA)/eicosapentaenoic acid (EPA) is administered together with FK506. Treatment with DHA/EPA and FK506 decreases the clinical score of dermatitis in NC/Nga mice and lowers local LTB4 concentrations. The treatment also suppressed the infiltration of T cells, B cells, eosinophils and neutrophils, and promoted reduced serum IgE levels. Secretion of IL-13 and IL-17A in $CD4^+$ T cells was lower in DHA/EPA- and FK506-treated mice than in mice treated with FK506 alone. The inhibition of disease progression induced by DHA/EPA was reversed by local injection of LTB4, suggesting that the therapeutic effect of DHA/EPA is LTB4-dependent. Our results demonstrate that treatment of AD with DHA/EPA is effective for allergic skin inflammation and acts by suppressing LTB4 production. J. Med. Invest. 63 : 187-191, August, 2016

Keywords **:** *docosahexaenoic acid, eicosapentaenoic acid, atopic dermatitis, leukotriene B4*

INTRODUCTION

Atopic dermatitis (AD) is caused by a dysregulated immune response and skin barrier disruption (1-4). AD patients generally have elevated IgE as well as enhanced differentiation of Th1, Th2, and Th17 cells (2, 5). Moreover, increased numbers of Th17 cells are associated with AD severity (6). As for skin barrier disruptions, loss-of-function mutations of filaggrin are associated with AD (7, 8) and filaggrin-deficient mice develop skin inflammation that is accompanied by increased Th17 differentiation (3, 9). Therefore, depending on disease severity, AD treatment can involve a combination of oral anti-histamines, anti-leukotrienes and ointments that include steroids, FK506 or NSAIDs (1). However, the long-term use of steroids or FK506 may cause severe side effects, which is problematic for younger patients in particular.

Several studies have shown that ω3 free fatty acid (FFA) intake through supplementation or foods can suppress the progression of inflammatory diseases (10, 11). These FFAs could suppress inflammatory responses by inhibiting production of IL-6 as well as prostaglandins and leukotrienes (12) that participate in skin inflammation by activating T cells and neutrophils (13). Moreover, the application of the leukotriene B4 (LTB4) receptor antagonist ONO-4057 inhibited spontaneous itch phenotypes in NC mice that are an animal model of AD (14). This finding suggests that LTB4 plays an important role in AD progression.

We developed an ointment containing the FFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) as a topical AD therapeutic and tested its efficacy in suppressing symptoms in AD patients (15). To determine the molecular mechanism by which this ointment alleviates symptoms, we evaluated the therapeutic efficacy of DHA/EPA on AD-like disease in NC/Nga mice. We found that DHA/EPA administered together with FK506 can ameliorate AD by suppressing LTB4 production. These data demonstrate the molecular basis for the therapeutic efficacy of DHA/EPA on AD and suggest that DHA/EPA may be a useful component in ointments used to treat AD.

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MATERIAL AND METHODS

Mice

NC/Nga mice (8-10 weeks old) were purchased from Charles River (Yokohama, Japan) and housed in the Animal Research Center of the University of Tokushima under specific pathogen-free conditions. All mice were housed under specific pathogen-free conditions. The animal studies in this manuscript was approved by the Committee on the Ethics of Animal Experiments of Tokushima University and the care and use of animals complied with institutional guidelines. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

DHA/EPA

Cod liver oil containing DHA 20% and EPA 10% were provided by NOF Corporation (Tokyo, Japan).

Dermatitis model

The hair in the rostral region on the backs of the mice was removed with a depilatory cream. The mice were then challenged with an ointment that included a *Dermatophagoides farinae* body extract (Biosta AD) (Biostir, Kobe, Japan) twice weekly for 3 weeks. The first day for the challenge was designated as day 0. Before the first challenge, the skin barrier was disrupted by treatment with a 4% SDS solution. The FK506 (0.3 mg) and/or DHA/EPA (Cod liver oil : 100 mg, EPA 8.3%, DHA 14.6%) ointment was applied at the area where Biosta AD was painted on days 4, 8, 12, 16, and 20.

Purification of cells from skin tissue and flow cytometry analysis

Skin tissue was digested with 2.5 mg/mL dispase II (Roche), 3 mg/ml collagenase (Worthington Biochemicals, USA) and 5 μg DNase (Roche) for 120 min at 37°C. After removal of cell debris, cells were stained with APC-conjugated anti-CD3, FITC-conjugated anti-B220, PE-Cy7-conjugated Gr1, APC-Cy7-conjugated CD11b, or PE-conjugated Siglec-F antibody. Flow cytometry was performed on a FCAS CantoII flow cytometer (Becton Dickinson, Mountain View, CA).

Cell culture

$CD4^+$ T cells were isolated from lymph nodes by incubating cells with anti-B220, anti-CD32/16, anti-CD11b, and anti-CD8 mAbs followed by incubation with anti-rat IgG-coated Dynabeads (Thermo Fisher Scientific Inc., USA). Purified $CD4^+$ T cells were stimulated with irradiated spleen cells in the presence of anti-CD3 mAb (1μg/ml) for 2 days.

Measurement of leukotriene levels

A section of skin (2 cm in diameter) was removed, minced with scissors and placed in ethanol containing indomethacin and zileuton. After sample homogenization and subsequent centrifugation for 5 min at 600×*g* and 4°C, the supernatant was mixed with water and applied to a C18 Sep-Pak cartridge (Waters, Milford, MA, USA) equilibrated with methanol. After washing the cartridge with hexane and then water, lipids were eluted with ethanol. The ethanol was evaporated, with the remaining residue suspended in an enzyme immunoassay buffer included with a LTB4 ELISA assay kit (Cayman Chemical, Ann Arbor, MI, USA). The protein concentration was measured and the amount of LTB4 was normalized relative to that of the overall protein.

ELISA

IL-13, IFN-γ, IL-17A, and IL-22 levels in culture supernatants were measured using an ELISA kit (R&D Systems, Minneapolis, MN). Serum from mice or standard IgG1, IgM, IgG2b or IgE (Southern Biotech, AL, USA) were serially diluted with PBS to coat a 96 well plate overnight at 4°C. After washing with PBS/0.1% Tween 20, alkaline phosphatase-conjugated goat anti-mouse IgG1, IgM, IgG2b, or IgE (Southern Biotech, AL, USA) were added and incubated at room temperature for 2 hours. After washing wells with PBS/0.1% Tween 20, alkaline phosphatase activity was determined using 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich, MO, USA) as the substrate.

Scoring of skin lesions

The extent of (1) erythema/hemorrhage, (2) scarring/dryness, (3) edema, and (4) excoriation/erosion was scored as 0 (none), 1 (mild), 2 (moderate), and 3 (severe). The total skin score was defined as the sum of the individual scores±SD.

Histology

Skin tissue samples were fixed in 10% formalin, embedded in paraffin, and sectioned. Tissue sections were stained with hematoxylin and eosin.

Statistical analysis

The distributed data from interval scales were analyzed with Student's *t*-test ; a *p* value of less than 0.05 was considered statistically significant.

RESULTS

DHA/EPA attenuate atopic dermatitis-like diseases

We sought to analyze the effect of DHA/EPA on atopic-dermatitis like diseases (AD) in NC/Nga mice, an animal model of AD. AD is induced in these mice by painting an ointment containing Dermatophagoides farinae body extract on their backs. We applied DHA/EPA or FK506 alone or DHA/EPA with FK506 to treat the AD. Treatment with DHA alone did not ameliorate AD as evaluated by clinical score and histology (Fig. 1a and b). Meanwhile, treatment with FK506 ameliorated AD symptoms, and treatment with both FK506 and DHA/EPA further improved the clinical scores and histology (Fig. 1a and b). These data demonstrate that DHA/

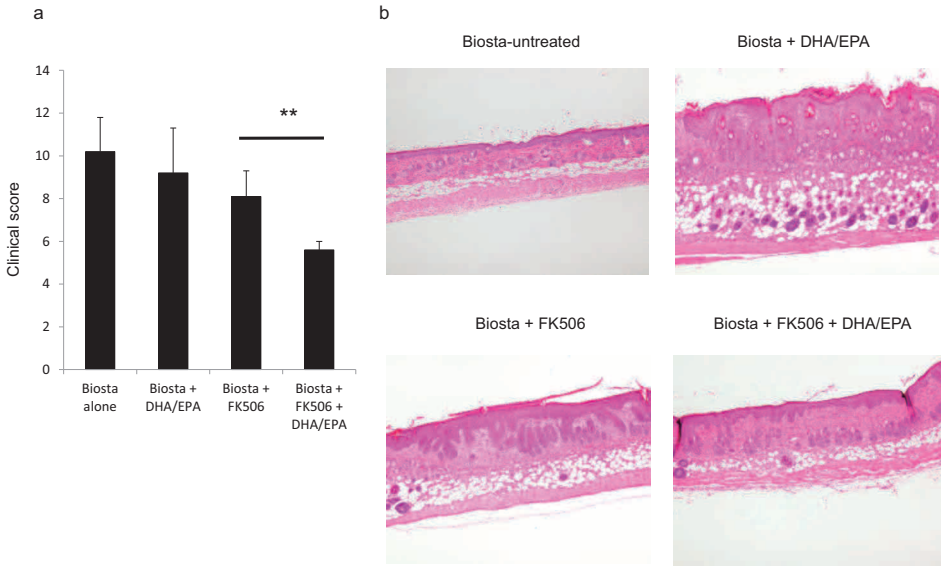

Figure 1. DHA/EPA attenuates skin inflammation
AD was induced by painting an ointment that contained *Dermatophagoides farinae* body extract onto the dorsal skin of NC/Nga mice. We applied DHA/EPA or FK506 or DHA/EPA with FK506 on the dorsal skin and evaluated the (a) clinical score and (b) skin histology by HE staining 30 days after initial induction. The data are shown as mean±S.D. (n=8 in each experiment). **indicates statistical difference ($p<0.01$).The data are representative of six independent experiments.

EPA can suppress AD when used with FK506.

DHA/EPA decreased the number of T cells that infiltrate the skin

We next evaluated the number of immune-related cells infiltrating the skin after treatment. Twenty days after initial AD induction, skin tissue samples were taken and digested with collagenase and dispase. The number of T cells ($CD3^+$), B cells (IgM^+B220^+), neutrophils ($CD11b^+Gr1^+$), and eosinophils ($CD11b^+SiglecF^+$) in these tissue samples were counted (Fig. 2). Treatment with FK506 did not affect cell numbers of any types of cells. In contrast, treatment with FK506 and DHA/EPA decreased the number of T cells, neutrophils and eosinophils (Fig. 2). These data show that DHA/EPA treatment could decrease the infiltration of T cells, neutrophils, and eosinophils in the skin.

DHA/EPA treatment decreases serum IgE and production of IL-13 and IL-17A by $CD4^+$ T cells

Given that DHA/EPA affects T cell numbers, we assessed serum immunoglobulin levels after DHA/EPA treatment (Fig. 3a). DHA/EPA together with FK506 decreased the amount of IgE but not levels of IgM, IgG1, and IgG2b. We also compared cytokine secretion from $CD4^+$ T cells in mice treated with FK506 alone and FK506 plus DHA/EPA. The $CD4^+$ T cells were purified from fluid drained from lymph nodes and stimulated with anti-CD3 mAb for 2 days. IL-13, IL-22, IL-17A, and IFN-γ expression was upregulated by treatment with *Dermatophagoides farinae* body extract (Fig. 3b). Treatment with FK506 and DHA/EPA decreased IL-13 and IL-17A secretion while FK506 alone did not affect the levels of any of the cytokines tested. Together these results indicated that DHA/EPA treatment decreases the secretion of IL-13 and IL-17A from $CD4^+$ T cells.

DHA/EPA treatment decreases LTB4 production

DHA/EPA have the capacity to suppress leukotriene production (16). We evaluated levels of the leukotriene LTB4 in the skin after DHA/EPA treatment. Dorsal skin tissue samples were digested and levels of the LTB4 protein were evaluated. FK506 treatment did not affect the amount of LTB4 in the skin, while FK506 with DHA/EPA significantly decreased LTB4 levels (Fig. 4a), indicating that DHA treatment affects LTB4 production.

To evaluate whether inhibition of LTB4 production contributes to the suppression of AD by DHA/EPA, we injected LTB4 into the skin of DHA/EPA-treated mice. LTB4 injection inhibited the effect of DHA/EPA ointment therapy (Fig. 4b), suggesting that DHA/EPA-mediated suppression of AD is attributable to the suppression of LTB4 production.

DISCUSSION

Bioactive lipids such as leukotrienes, prostaglandin, and eicosanoids are generated from arachidonic acid by 5-lipoxygenase and cyclooxygenase (17, 18). Leukotrienes and prostaglandins are involved in a variety of inflammatory diseases, including skin inflammation (17, 19). Based on our earlier finding that DHA could suppress symptoms in AD patients, here we used a mouse model of AD to assess whether an ointment containing DHA/EPA had therapeutic effects and also to determine the mechanisms by which DHA acts (15). Treatment with DHA/EPA alone had no effect on AD pathology, but treatment with both FK506 and DHA had strong therapeutic potential in the mouse model of AD. Furthermore, the effect of DHA/EPA was dependent on inhibition of LTB4 production. These data suggest the DHA may suppress symptoms of AD by inhibiting the production of key inflammatory eicosanoids.

Previous studies have suggested that the effect of FFA on inhibiting inflammation is attributable to changes in the fatty acid composition of cellular phospholipids, since FFAs rather than arachidonic acid can incorporate into cell membranes (20). Because FFAs are poor substrates for 5-lipoxygenase and cyclooxygenase, FFA incorporation into cell membranes could in turn suppress the production of eicosanoid mediators (16). The inhibition of LTB4 production by DHA/EPA treatment in our study also suggested the inhibitory effect of DHA/EPA on arachidonic acid. Alternatively, DHA and other FFAs could activate the fatty acid receptor GPR120 on cell surfaces, which may subsequently inhibit phosphorylation of the transforming growth factor β-activated kinase 1 (TAK1) that participates in the NF-kB and MAPK signaling pathways (21). Given that inhibition of TAK1 by RNAi suppresses Th1 and Th17 differentiation by targeting myeloid cells (22), future studies on the effect of DHA on TAK1 phosphorylation levels *in vivo* would be important to determine if TAK1 is associated with DHA/EPA-mediated suppression of AD pathology.

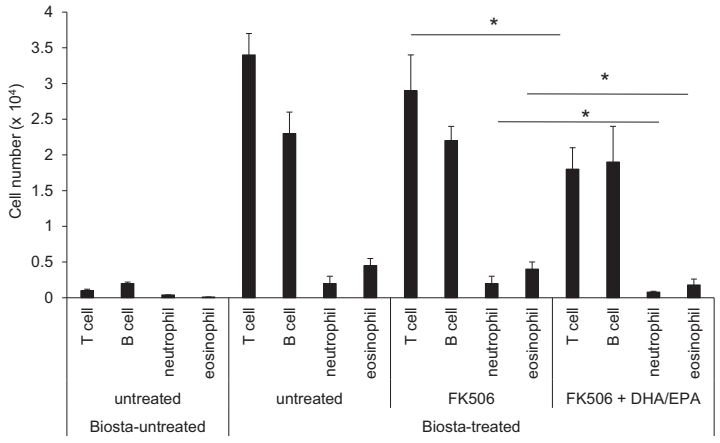

Figure 2. DHA/EPA treatment decreases T cell and eosinophil numbers in skin tissue
Dorsal skin tissue from NC/Nga mice treated with DHA/EPA, FK506 or DHA/EPA with FK506 (20 days after initial induction) was digested by collagenase and dispase. The cell numbers were then counted and the cells were stained with antibodies against CD3, IgM, B220, CD11b, Gr1 or SiglecF. The number of T cells ($CD3^+$), B cells (IgM^+B220^+), neutrophils ($CD11b^+Gr1^+$), and eosinophils ($CD11b^+SiglecF^+$) are expressed as a total cell number/g of skin tissue. The data are shown as mean±S.D. (n=8 in each experiment). **indicates statistical difference ($p<0.01$).The data are representative of five independent experiments.

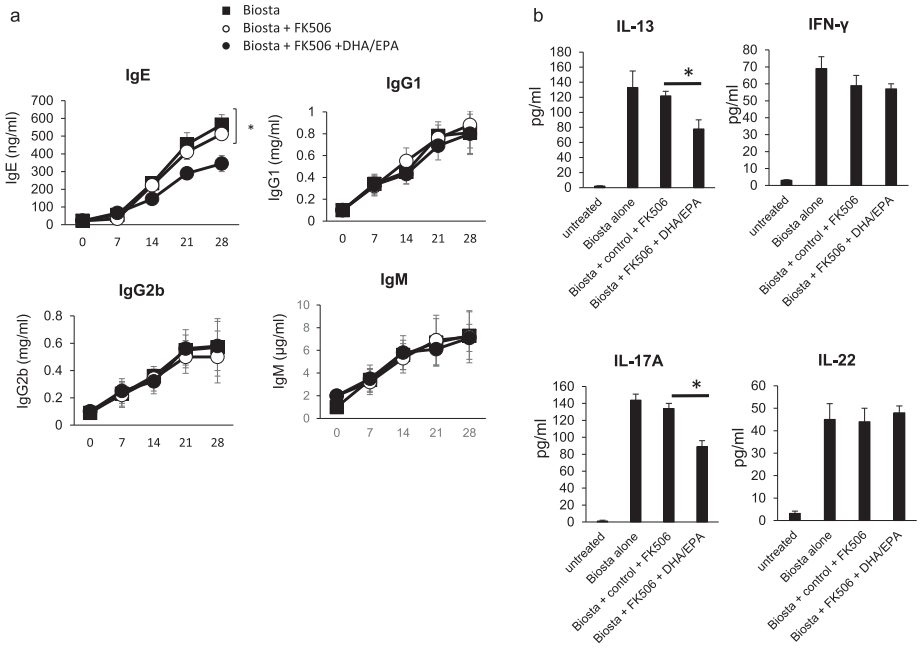

Figure 3. DHA/EPA treatment decreases IgE and cytokines expression from T cells
(a) AD was induced by painting an ointment containing *Dermatophagoides farinae* body extract onto the dorsal skin of NC/Nga mice. We painted FK506 alone or DHA/EPA with FK506 on the dorsal skin of mice and evaluated serum IgE, IgG1, IgG2, and IgM levels 30 days after initial induction. The data are shown as mean±S.D. (n=8 in each experiment). *indicates statistical difference ($p<0.05$). (b) We applied FK506 alone or DHA with FK506 on the dorsal skin of mice treated with Biosta. Thirteen days after initial Biosta treatment, $CD4^+$ T cells were purified and stimulated with plate-coated anti-CD3 mAb for 2 days. Levels of IL-13, IL-17A, IL-22, and IFN-γ in the supernatant were evaluated by ELISA. The data are shown as mean±S.D. (n=8 in each experiment). *indicates statistical difference ($p<0.05$). The data are representative of six independent experiments.

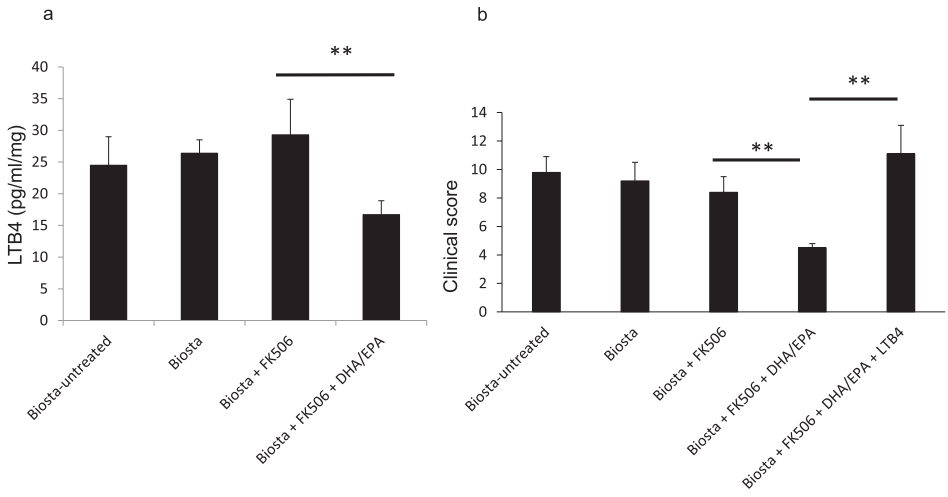

Figure 4. The therapeutic effect of DHA/EPA is attributable to the suppression of LTB4
AD is induced by painting an ointment containing *Dermatophagoides farinae* body extract onto the dorsal skin of NC/Nga mice. (a) We applied DHA/EPA or FK506 alone or DHA with FK506 onto the dorsal skin and collected dorsal skin tissue 20 days after initial induction. LTB4 protein levels were measured by ELISA. The data are shown as mean±S.D (n=8 in each experiment). *indicates statistical difference ($p<0.05$). (b) We applied FK506 alone or DHA/EPA with FK506 onto the dorsal skin and then injected LTB4 at 10 different sites on the dorsal skin (2 pg/site). The clinical score was evaluated. The data are shown as mean±S.D. (n=5 in each experiment). *indicates statistical difference ($p<0.05$).The data are representative of three independent experiments.

The therapeutic efficacy of DHA/EPA was observed when it was administered together with FK506 while DHA/EPA alone failed to ameliorate AD pathology. FK506 is a potent T cell suppressive drug that makes a complex with FKBP resulting in inhibiting dephosphorylation of NFAT (23). This finding may have two possible explanations : i) treatment with DHA/EPA alone may be able to suppress AD, but its effect would be too weak to ameliorate AD pathology in this AD model ; or ii) Suppression of T cell responses by FK506 is needed in order for DHA/EPA to exert its effects in this model. These AD model mice exhibited a scratching behavior that is related to the progression of AD pathology. FK506 treatment suppressed this scratching behavior (24), and thus this

compound might be required for DHA/EPA to produce a therapeutic effect on AD. In contrast, in AD patients DHA/EPA alone has treatment efficacy, which might be attributable to the lower incidence of scratching in humans relative to NC/Nga mice.

In conclusion, DHA/EPA could suppress AD pathology by inhibiting LTB4 production in an AD mouse mode. Although the molecular mechanism by which DHA/EPA suppresses LTB4 in this AD model awaits further clarification, these data suggest that ointments including DHA/EPA would be a beneficial treatment for AD patients when DHA/EPA is administered together with FK 506.

CONFLICT OF INTERESTS-DISCLOSURE

All authors do not have any conflict of interest.

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