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Association between very long chain fatty acids in the meibomian gland and dry eye resulting from n-3 fatty acid deficiency

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ABSTRACT

In our previously study, we reported lower tear volume in with an n-3 fatty acid deficient mice and that the docosahexaenoic acid and total n-3 fatty acid levels in these mice are significantly reduced in the meibomian gland, which secretes an oily tear product. Furthermore, we noted very long chain fatty acids (≥ 25 carbons) in the meibomian gland. To verify the detailed mechanism of the low tear volume in the n-3 fatty acid-deficient mice, we identified the very long chain fatty acids in the meibomian gland, measured the fatty acid composition in the tear product. Very long chain fatty acids were found to exist as monoesters. In particular, very long chain fatty acids with 25–29 carbons existed for the most part as iso or anteiso branched-chain fatty acids. n-3 fatty acid deficiency was decreased the amount of meibum secretion from meibomian gland without change of fatty acid composition. These results suggest that the n-3 fatty acid deficiency causes the enhancement of evaporation of tear film by reducing oily tear secretion along with the decrease of meibomian gland function.

1. Introduction

Docosahexaenoic acid (DHA; 22:6n-3) is an n-3 polyunsaturated fatty acid that plays an important role in nervous system function and accumulates in the brain. Decreased levels of n-3 fatty acids in the brain have been reported to affect learning ability, cognitive function, and emotional behavior [1–3]. In addition, DHA can pass through the blood–retinal barrier and constitutes about 50–60% of the fatty acid composition of retina [4], which converts light stimuli to neural signals and transmits them to the brain via the optic nerve [5,6]. Thus, the retina is the most important component of the eye for visual function, and DHA is critical for visual function due to its role in activating retina signaling. Several studies have reported that low levels of retinal DHA reduce visual function. In addition, the secretory organs located around the eye maintain visual function. Specifically, the eye secretes tears as protection against external environmental factors such as foreign objects and from internal processes such as desiccation. Tears provide a stable supply of a 3-layered fluid comprising mucin, water and oil to the ocular surface through the normal secretory functions of the conjunctiva, lacrimal gland, and meibomian gland, respectively. Dry eye syndrome in a common eye condition leading to abnormalities in visual function and ocular discomfort due to the lack of tears and consists of two types. One is where the secretion of aqueous tear production is reduced, and the other is where the aqueous tear product evaporates as a result of decreased secretion of oily tear product. The meibomian gland secretes an oily tear product to the outermost layer that prevents this evaporation of the aqueous material.

Our previous murine study reported that n-3 fatty acid deficiency results in reversible dry eye syndrome and, moreover, a decreased quantity of n-3 fatty acids and total fatty acids in the meibomian gland [7]. And, dry eye symptoms were almost completely ameliorated after oral administration of fish oil containing eicosapentaenoic acid (EPA; 20:5n-3) and DHA. Furthermore, fatty acids with ≥ 24 carbons (known as very long chain fatty acids; VLCFAs) were found to constitute about 30% of the total fatty acids in the meibomian gland. However, few researches have been performed on the association between n-3 fatty acids and meibomian gland function. In the present study, we used GC and GC–MS to identify the structure of the unsaturated fatty acids in the meibomian gland and the location of their double bonds.

Abbreviations: VLCFAs, very long chain fatty acids; BCFAs, branched chain fatty acids; DMOX, 4, 4-dimethyloxazoline; DHA, docosahexaenoic acid; n-3 Def, n-3 fatty acid deficiency; n-3 Adq, n-3 fatty acid adequacy; TLC, thin-layer chromatography; MS, mass spectrometry; GC, gas chromatography; FAMEs, fatty acid methyl esters

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And we examined the relationship between dry eye symptoms and fatty acid levels by identifying the levels of VLCFAs in the meibomian gland containing tear product.

2. Material and methods

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Azabu University.

2.1. Animals and diets

Two experimental diets, an n-3 fatty acid deficient (n-3 Def) diet and an n-3 fatty acid adequate (n-3 Adq) diet were based on the AIN-93G dietary recommendations for rodents (Oriental Yeast Co, Tokyo, Japan, Table 1). The fat content in both diets was 7% (w/w) and consisted mainly of hydrogenated coconut and safflower oils. The major difference between the experimental diets was the concentration of n-3 fatty acids. Flaxseed oil, which is rich in alpha-linolenic acid (18:3n-3), was added to the n-3 Adq diet. The concentration of the n-3 fatty acids of the total fatty acids in the n-3 Def and n-3 Adq diets was 0.25% and 2.5%, respectively. The level of n-6 fatty acids, which consisted of only linoleic acid (18:2n-6), was the same in both diets (14.6%). CD-1 (ICR) mice were obtained from Charles River Japan, Inc. (Yokohama, Japan) mated, and fed either type of diet. Male offspring were maintained on same diet of their dams, and their tear production was collected at 45 weeks old. After that, their meibomian glands were cut out. All mice were bred within our animal facility under conventional conditions with controlled temperature (23 ± 3 °C), humidity (55 ± 10%), and illumination (12 h; 07:00–19:00).

2.2. Thin-layer chromatography of lipid content in meibomian gland extract

Each meibomian gland of mouse was homogenized with 0.9% NaCl. Total lipid was extracted from whole tissues of the upper and lower meibomian gland homogenate according to the method of Bligh and Dyer [8]. The samples were dissolved in 100 μl chloroform and the extracted lipids were separated by thin-layer chromatography (TLC). The samples were then spotted on a TLC Silica gel 60 glass plate (E. Merck, Darmstadt, Germany) with an eluent composition of pentane: diethylether:acetic acid of 70:30:1. TLC separations were run for non-polar lipid, triglyceride, monoester, and free cholesterol. After separation, the plates were stained with iodine vapor, and the separated lipids were scraped from the TLC plate, transmethylated, and derivatived.

2.3. Lipid extraction and transmethylation

The transmethylation method developed by Lepage and Roy was used [9]. An internal standard solution (22:3n-3 methyl ester) and each separated lipid (polar lipid, triglyceride and monoester) was added to borosilicate glass tubes (13 mm × 100 mm) containing 2 ml of a methanol-hexane mixture (4:1, v/v) with 50 μg/ml of butylated hydroxytoluene to prevent lipid oxidation during the procedures, and the tubes were placed on ice. Acetyl chloride of 200 μl was added while swirling the tubes. The tubes were capped under a nitrogen gas and transferred to a heating block at 100 °C for 60 min. Then the samples were placed on ice and added 5 ml of a 6% solution of K_2CO_3. The tubes were vortexed and then centrifuged for 15 min at $2200 \times g$ to remove emulsion and separate the mixture into two phases. The upper phase of hexane was collected, and the hexane extracts were concentrated in micro-vials for GC injection.

Table 1
Composition of experimental diets.

	Amount (g/100 g diet)	
	n-3 Adq.	n-3 Def.
Casein, vitamin free	20	20
Carbohydrate:	63	63
Cornstarch	15	15
Sucrose	13	13
Glucose	20	20
Dextrose	7.5	7.5
Maltose	7.5	7.5
Cellulose	5	5
Mineral-Salt mix	3.5	3.5
Vitamin mix	1	1
L-Cystine	0.3	0.3
Choline bitartrate	0.25	0.25
TBHQ	0.002	0.002
Fat	7	7
Hydrogenated coconut oil	5.43	5.67
Safflower oil	1.24	1.33
Flaxseed oil	0.34	none
Fatty acid composition (% of total fatty acids)		
Saturates	75.1	78.1
Monounsaturates	5.4	4.7
18:2n-6	14.6	14.6
18:3n-3	2.5	0.25
n-6/n-3	5.8	58.4

The two experimental diets, an n-3 fatty acid adequate diet (n-3 Adq) and an n-3 fatty acid deficient diet (n-3 Def), were based on the AIN-93 formulation with several modifications to obtain the extremely low basal level of n-3 fatty acid required in this study.

2.4. Preparation of fatty acid methyl esters, 4,4-dimethyloxazoline derivatives, and picolinyl derivatives

Fatty acid methyl esters (FAMEs) for analysis of the VLCFAs were purified by TLC and analyzed by GC. The derivatives of DMOX and picolinyl esters can detect double bond and branching positions by GC–MS [10,11]. These were prepared from FAMEs and natural lipids. FAMEs were converted to DMOX derivatives by treatment with 200 μl 2-amino-2-methyl-1-propanol in a test tube at 180 °C for 12 h under a nitrogen gas [10]. The reaction mixture was cooled and dissolved in 500 μl dichloromethane:hexane (1:1) and further washed twice with 2 ml water. The solvent was removed under a stream of nitrogen gas and the sample was dissolved in hexane for GC–MS analysis. Picolinyl esters were prepared in dry dichloromethane of 500 μl mixed with a solution of potassium tert-butoxide in tetrahydrofuran and 3-hydroxymethyl pyridine of 100 μl (1:2, v/v) according to the method of Destaillats and Angers [11]. The mixture was held at 45 °C for 45 min in a closed vial. After cooling to room temperature, water and hexane were added, and the organic phases were collected, dried over anhydrous sodium sulfate, and evaporated. The sample was finally dissolved in hexane for GC–MS analysis. These deris

2.5. GC analysis

FAMEs were analyzed using an Agilent 7890 A Network Gas Chromatograph (Agilent Technologies; Palo Alto, CA) equipped with a split injector, a 7693A automatic liquid sampler, an FID, and a 208 V power supply to enable rapid temperature ramping. The instrument was controlled, and data were collected using GC Chemstation Rev.B0.040.01.SP1 (Agilent Technologies). The column was used a DB-FFAP (15 m × 0.1 mm i.d. × 0.1 μm film thickness, J&W Scientific, Agilent Technologies). The detector and injector temperatures were set to 250 °C. The oven temperature program began at 150 °C with a 0.25 min hold, was ramped at 35 °C/min to 200 °C followed by 8 °C/min to 225 °C with a 3.2 min hold and