

Note

Influence of Hot Spring Water on Fatty Acid Composition of Skin Surface Lipids in Hairless Mouse Model of Atopic Dermatitis

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When hairless NCN24 mice with atopic dermatitis (AD) were sprayed with a petroleum-containing alkaline salt spring water rich in metaboric acid and sodium bicarbonate, AD symptoms diminished. Reversed-phase HPLC with fluorescence detection (HPLC/FD) and online MS revealed that fatty acid (FA) composition of the skin surface lipids was similar to that in non-AD mice compared with that in AD mice. Strong negative correlations were noted between the levels of total serum immunoglobulin E (IgE) and palmitoleic acid and between the levels of total serum IgE and branched-hexadecanoic acid. Conversely, a strong positive correlation was noted between the levels of total serum IgE and linoleic acid. The present study demonstrates that the petroleum-containing spring water alters the FA composition of skin surface lipids in AD mice, which can be used as an index to evaluate inflammation.

Key words atopic dermatitis; hot spring water; fatty acid; NCN24 mouse; skin surface lipid; fluorescence detection

Atopic dermatitis (AD) is a common inflammatory skin disease that has both genetic and environmental factors in its etiology.¹⁾ It is well known that lipids, fatty acids (FAs) and their metabolites play an important role in the control of inflammation in AD.²⁾ There are many reports on the relation between FA composition and inflammation. For example, an abnormal *n*-6 FA composition in cheek mucosal cells of children with AD³⁾ and an elevation of serum linoleic acid (18:2n-6) level in children with AD compared with that in non-AD children⁴⁾ have been observed in studies on AD and lipid metabolism. It has also been observed that arachidonic acid (20:4n-6) significantly accumulated in blood cells of NC/Nga mice (an AD animal model) with AD.²⁾

Using NC/Nga mice,^{5,6)} we had previously investigated methods for assessing balneotherapy effects on pathogenesis of AD, in which the dermatitis score was used as an index to evaluate inflammation.^{7,8)} Comprehensive analysis of gene expression in NC/Nga mice with AD revealed significant changes in expression of several genes involved in fatty acid metabolism, such as acetyl CoA carboxylase.⁸⁾ To our knowledge, however, there have been no reports on FA composition of the skin surface lipids that play an important role in moisturizing and the skin barrier system in AD mouse models. This may be due to difficulty in lipid extraction from mice with hair such as NC/Nga mice.

In the present study, we investigated the influence of spring water on the FA composition of skin surface lipids by using a hairless AD mouse model, NCN24 mice (transgenic mice)⁹⁾ and reversed-phase HPLC with fluorescence detection (HPLC/FD).^{10,11)} The spring water examined was available from Toyotomi hot spring (Hokkaido, Japan) that is famous for being effective against AD and psoriasis, and many people from across the country has visited the watering for cure.¹²⁾ One of the features of the spring is to include petroleum-containing hot spring water that is rare in Japan. Although there is not enough scientific evidence to support the effect of the hot spring water, certain petroleum components may be involved,

since it has been reported that topical application of coal tar is an effective AD therapy for reducing inflammation and itch, and has been used to treat skin diseases for more than 2000 years.¹⁾

We sprayed the spring water on AD mice (S group) and determined FA composition of skin surface lipids in mice in which skin symptoms decreased after treatment and in mice sprayed with distilled water (D group). Here we describe significant differences in FA composition of skin surface lipids observed between the groups, suggesting that FA composition of skin surface lipids can be used as an index to evaluate AD.

MATERIALS AND METHODS

Materials HPLC-grade solvents, methanol and acetonitrile, and ultrapure water were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 9-Anthryldiazomethane (ADAM) was purchased from Funakoshi Co. (Tokyo, Japan). Branched-chain FA standards, isopentadecanoic (15:0b), isopalmitic (16:0b), isostearic (18:0b), and isononadecanoic (19:0b) acids, and normal-chain saturated and unsaturated FA standards with C14–C22 and 0–6 double bonds were obtained from Sigma (St. Louis, MO, U.S.A.). Individual FAs were dissolved in methanol or chloroform to obtain 10 mg/mL standard solutions, which were used for HPLC and HPLC/MS after dilution with methanol.

Hot Spring Water Petroleum-containing alkaline salt hot spring water rich in metaboric acid and sodium bicarbonate (pH 8.1) was collected once a week from Toyotomi hot spring (Toyotomi-cho, Hokkaido, Japan). After the surface oil was eliminated by decantation, the spring water was sterilized by filtration through a 0.20- μ m filter (Nalgen). The chemical composition of the spring water, which was determined based on the Standard Methods of Analysis for Mineral Springs published by Ministry of the Environment-Government of Japan,¹³⁾ were as follows (in mg/kg): Na⁺ 4683, K⁺ 25.3, Mg²⁺ 19.2, Ca²⁺ 16.4, Cl[–] 6242, HCO₃^{3–} 2514, SO₄^{2–} 0.0, Br[–] 27.1, I[–]

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14.7, and HBO_2 449.6, and dissolved matter content (excluding gaseous substances) was 12.58 g/kg. Pure water prepared from distilled water using Simplicity™ Water Purification System (Merck Millipore) was sterilized by filtration and used as a reference.

Hairless NCN24 Mice Eight male mice (age, approximately 12 weeks) sensitized and challenged with 2,4-dinitrofluorobenzene (DNFB; 0.15% in acetone) injections in the cervical region under specific pathogen free (SPF) conditions to induce dermatitis and 3 mice without sensitization or dermatitis induction as a control group were purchased from Immuno-Biological Laboratories Co. (Fujioka, Japan). The 8 mice were divided into D and S groups immediately before the study to standardize the dermatitis score and serum immunoglobulin E (IgE) levels between the groups. The study duration was 3 weeks, and dermatitis was induced by challenging with 0.15% DNFB twice a week throughout the study period. The D and S groups were treated with distilled and spring water twice a day at room temperature, respectively, and skin symptoms were evaluated as described by Lee *et al.*¹⁴ Briefly, three AD-like symptoms (dryness, crust, keratinization) were scored into four graded severities from 0 (none) to 3 (severe). Total serum IgE was measured using a mouse IgE measurement EIA kit (Yamasa Co., Chiba, Japan). This study was approved by the Ethics Committee for Experimental Animal of the Hokkaido Institute of Public Health.

Preparation of Fluorescent Derivatives Absorbent cotton washed with chloroform and dried beforehand was immersed in hexane, and skin lipids were collected from the animals by employing the wet wiping method on the final day.¹⁵ After non-lipids were eliminated from the hexane extracts (2–4 mg) by Folch partition, the residual lipids were hydrolyzed to FAs by heating at 100°C for 45 min in a 5 M HCl/CH₃OH–H₂O (9:1, v/v) solution.¹⁶ The 9-anthrylmethyl ester derivatives of FAs were prepared using 9-anthryldiazomethane (ADAM).¹⁰

HPLC/FD and HPLC/MS HPLC/FD was performed with a Hitachi L-7000 series comprising a pump, a degasser, a fluorescence detector, an autosampler, and an oven (Hitachi, Tokyo, Japan). Analysis was performed on an X-Terra MS C18 column (150×3.0 mm i.d., 3.5- μm particles, Nihon Waters, Tokyo, Japan) at 50°C using an isocratic elution with water–methanol (8:9, v/v) at a flow rate of 0.3 mL/min. Sample solution (10 μL) was injected into the column, and effluents were monitored at 365/412 nm (Ex/Em).¹⁷ HPLC-MS was performed on a Shimadzu LCMS-2010EV (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) interface. The probe voltage, curved desolvation line (CDL) temperature, CDL voltage, and nitrogen gas flow rate were 4.5 kV, 250°C, 25 V, and 4.5 L/min, respectively.¹¹ HPLC conditions were the same as those described above.

Statistical Analysis Statistical analysis was performed using Dr. SPSSII (SPSS Japan Inc., Tokyo). *p* Values of <0.05 were considered significant. For comparisons of the 3 groups, ANOVA was used. When ANOVA revealed significant differences, Scheffe's test was used as a *post hoc* test.

RESULTS AND DISCUSSION

Total Serum IgE Level and Dermatitis Score On the final day of the 3-week study period, total serum IgE level and

dermatitis score were 943.7 ng/mL and 3, respectively, in the D group, which were significantly higher than those (284.4 ng/mL and 1, respectively) in the S group. These parameters were significantly reduced compared with those on the first day in S group, confirming the effects of spring water. Figure 1 shows changes in total serum IgE levels over the study period, and Table 1 gives the dermatitis score and total serum IgE level on the final day. A correlation was noted ($R=0.957$) between the dermatitis score and total serum IgE level, and AD-like symptoms were observable visually and in serum cytokine responses in mice that developed dermatitis (Fig. 2). The D group mice revealed skin lesions characterized by dryness, crust, keratinization, and erythema, whereas in S group mice the AD symptom was almost disappeared (Fig. 1S).

Fatty Acid Profiles of Skin Surface Lipids Figure 3 shows the reversed-phase HPLC chromatogram of the 9-anthrylmethyl ester derivatives of FAs of skin surface lipids collected from a mouse in the S group on the final day. Although no effective resolution of double-bond positional isomers and iso/anteiso isomers was obtained, use of a hairless mouse model (NCN24 mice) and HPLC/FD enabled the measurement of FAs in skin surface lipids of individual mice.

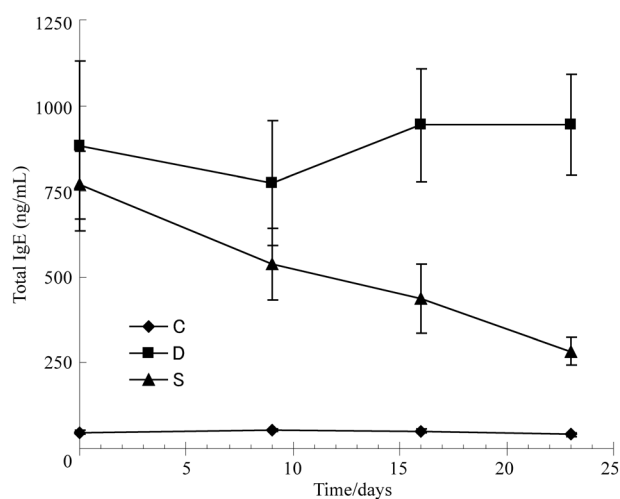


Fig. 1. Total Serum IgE Levels of Mice over the Study Period

◆: control (mice with non-atopic dermatitis, C group), ▲: spring water treated mice (S group), ■: distilled water treated mice (D group).

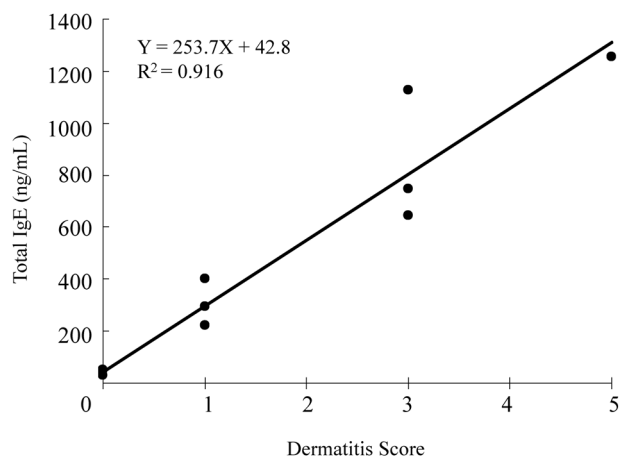


Fig. 2. Total Serum IgE Levels and Dermatitis Score for All the Mice Examined

Table 1. Total Serum IgE, Dermatitis Score and Fatty Acid Compositions of Skin Surface Lipids (Sebum) of NCN24 Hairless Mouse Model of Atopic Dermatitis

	Control (C)	Mouse of atopic dermatitis		Multiple comparison ^{a)}
		Treated with distilled water (D)	Treated with spring water (S)	
Total IgE (ng/mL) ^{b)}	41.33±6.87	943.71±146.70	284.43±41.87	C-D, D-S**
Dermatitis score ^{c)}	0 (0-0)	3 (3-5)	1 (1-1)	C-D**
Fatty acid (mol%) ^{b,d)}				
14:0	1.71±0.20	2.09±0.17	1.91±0.21	
16:0	7.04±0.21	7.29±0.69	7.56±0.87	
18:0	3.82±0.08	5.72±0.42	4.07±0.04	C-D, D-S**
20:0	7.59±0.17	6.19±0.18	6.19±0.24	C-D, C-S**
21:0	0.74±0.01	0.67±0.01	0.64±0.07	
22:0	5.43±0.55	5.31±0.08	4.53±0.33	
Σ Saturated	26.32±0.95	27.26±1.13	24.91±0.23	
16:1	9.34±0.41	3.69±0.41	5.50±0.14	C-D, C-S**, D-S*
18:1	15.59±0.72	19.57±0.53	16.36±0.42	C-D, D-S**
20:1	12.47±0.75	10.57±0.86	11.00±0.19	
22:1	11.06±0.61	12.49±0.23	13.45±0.40	C-S*
24:1	1.90±0.17	2.68±0.04	2.50±0.15	C-D**, C-S*
18:2	4.31±0.30	5.89±0.22	5.20±0.24	C-D**
Σ Unsaturated	54.67±1.07	54.89±0.96	54.01±0.18	
16:0b	1.64±0.06	0.98±0.08	1.52±0.05	C-D, D-S**
18:0b	0.81±0.06	0.70±0.06	0.77±0.05	
19:0b	1.98±0.21	1.68±0.10	2.34±0.11	D-S*
20:0b	5.07±0.14	4.96±0.10	5.90±0.15	C-D, D-S**
21:0b	4.09±0.21	3.82±0.10	4.63±0.07	D-S**
22:0b	5.42±0.48	5.70±0.20	5.92±0.30	
Σ Branched	19.01±0.77	17.85±0.23	21.08±0.21	D-S**, C-S*

a) Asterisks in the column indicate a significant difference among the three groups (* $p<0.05$; ** $p<0.01$). b) Values are the mean±S.E. ($n=3$ for C; $n=4$ for D and S). c) Dermatitis score is shown by median (range).

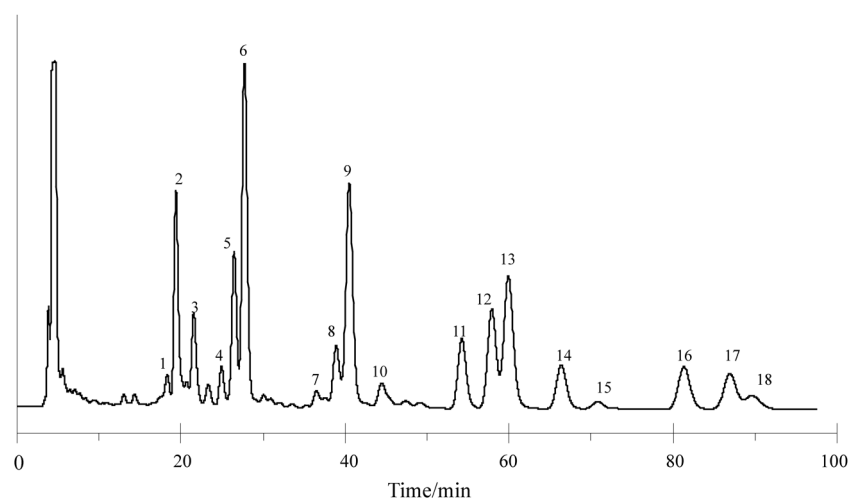


Fig. 3. Reversed-Phase HPLC/FD Profile of the 9-Anthrylmethyl Esters of Fatty Acids from the Skin Surface Lipids of One NCN24 Mouse (S Group)

Peak identification: 1=14:0, 2=16:1, 3=18:2, 4=16:0b, 5=16:0, 6=18:1, 7=18:0b, 8=18:0, 9=20:1, 10=19:0, 11=20:0b, 12=20:0, 13=22:1, 14=21:0b, 15=21:0, 16=22:0b, 17=22:0, 18=24:1. HPLC conditions are as given in the text.

Eighteen peaks were eluted with good separation. HPLC/MS analysis was performed to identify peaks 10–18, which were inconsistent with the retention times (RTs) of standards,¹⁷⁾ and a prominent (M+Na)⁺ ion was detected in each peak: m/z 512 was detected in peak 10, m/z 525 in peaks 11 and 12, m/z 551 in peak 13, m/z 539 in peaks 14 and 15, m/z 553 in peaks 16 and 17, and m/z 579 in peak 18. The FA 9-anthrylmethyl ester

derivatives are eluted in order of increasing equivalent carbon number (ECN = total carbon number – 2 × number of double bonds) in reversed-phase HPLC.¹⁸⁾ The logarithmic values of RTs of FA homologues, such as saturated, monounsaturated, and branched-chain FAs, exhibit a linear relationship with the carbon number.¹⁹⁾ Based on these relationships and MS findings, peaks 10–18 were identified to be 19:0, 22:0b, 22:0,

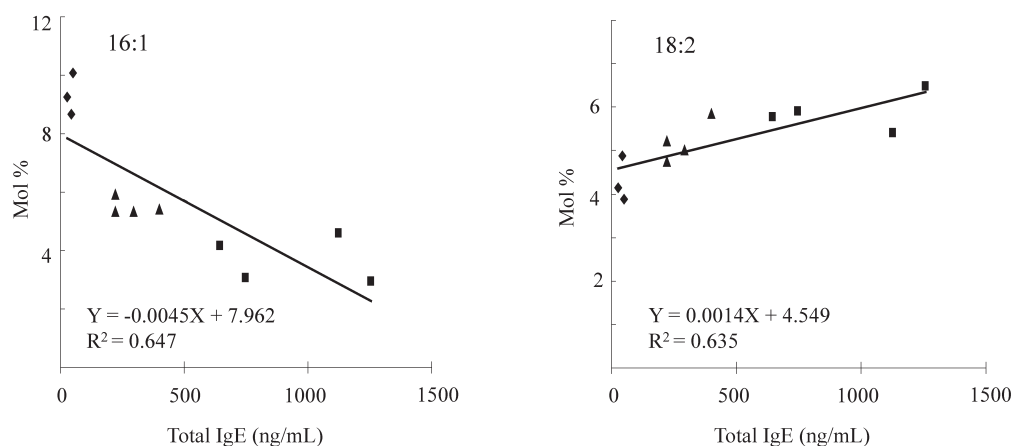


Fig. 4. Relationship between Total Serum IgE Levels and Percentages of 16:1 (Left Panel) and 18:2 (Right Panel) in the Skin Surface Lipids of Mice

◆: control (mice with non-atopic dermatitis, C group), ▲: spring water-treated mice (S group), ■: distilled water-treated mice (D group).

22:1, 21:0b, 21:0, 22:0b, 22:0, and 24:1, respectively.

Table 1 also shows the FA composition of skin surface lipids of NCN24 mice, exhibiting a pattern similar to that in experimental mice previously reported.²⁰⁾ Branched-chain FAs, not contained in serum (data not shown), accounted for approximately 20% of the total FA content and FAs with a carbon number of ≥ 20 accounted for approximately 50% of total FA content. The 18:2n-6 content (mol%), reported to be associated with AD and lipid metabolism,⁴⁻⁶⁾ decreased in the order of D group > S group > control group (C group), similar to that of total serum IgE level. The 18:0 and 18:1 contents also exhibited a similar tendency. Conversely, the content of 16:1, which may be associated with moisturizing effects by its strong lubricity,²¹⁾ decreased in the order of C group > S group > D group. The branched-chain FA content displayed an order similar to 16:1 content: C group > S group > D group, and as did 16:0b content. FA composition in the S group, in which skin symptoms diminished, was similar to that in the non-AD group, but not to that in the AD group.

Figure 4 illustrates the correlation between FA composition and total serum IgE level. The correlation coefficient between the 16:1 content and total serum IgE level was -0.805 and between 16:0b content and total serum IgE level was -0.919 , revealing strong negative correlations. In addition, the correlation between 18:2 content and total serum IgE level was 0.797 , revealing a strong positive correlation. As AD symptoms exacerbated, proportion of 18:2, involved in inflammation, increased, whereas that of 16:1 and 16:0b decreased. Reduction in proportions of 16:1 and 16:0b in human skin surface lipids with aging has been reported,²²⁾ suggesting that these FAs also play an important role in NCN24 mouse skin surface lipids. The 16:1 and 16:0b FAs are known to be the major constituents in mammalian skin surface lipids.²³⁾ Both acids exhibit bactericidal properties against Gram-positive bacteria, such as *Staphylococcus aureus* and *Staphylococcus salivarius*. It is now widely accepted that patients with AD are prone to cutaneous *Staphylococcus aureus* infection during phases of acute exacerbation and that an increased density of *S. aureus* is found to correlate well with the severity of skin manifestations.²⁴⁾ In preclinical and human epidemiological studies, 16:1 has shown anti-inflammatory effect.^{25,26)} The decrease of 16:1 and 16:0b might be caused by deficiency of

fatty acid synthases including desaturase²⁷⁾ and elongase,²⁸⁾ as indicated in essential FA metabolism.²⁹⁾ while in the increase of 18:2, a contamination of inflammatory cells infiltrated from blood might be involved, but no detection of this acid has been reported in the spring water.

CONCLUSION

In the present study, we investigated the influence of petroleum-containing hot spring water on the FA composition of skin surface lipids in hairless mouse model of AD and revealed for the first time an AD symptom-associated alteration of the FA composition. However, currently, it is unclear whether these variations aggravate AD symptoms or whether aggravation of AD symptoms alters FA composition. Further studies, including a comprehensive analysis of serum FAs, skin tissue lipids, and genes in samples collected from the same individuals, will be necessary to clarify the relationship between balneotherapy and FA metabolism.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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