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Selective Reduction in α-Hydroxypalmitic Acid-containing Sphingomyelin and Concurrent Increase in Hydroxylated Ceramides in Murine Skin Tumors Induced by an Initiation-promotion Regimen

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The sphingomyelin cycle is activated to accumulate ceramides in the process of epidermal differentiation. We found that sphingomyelin in the epidermis of 4 different murine strains gave three bands on TLC, the lower band containing α-hydroxypalmitic acid (C16h:0(α)). However, in the papillomas induced in the skin of SENCAR and SSIN mice by initiation with 7,12-dimethylbenz[*a*]anthracene followed by promotion with 12-*O*-tetradecanoylphorbol acetate, the concentration of C16h:0(α)-containing sphingomyelin was selectively diminished with a concomitant increase in the concentrations of the ceramides containing α-hydroxy fatty acids. These findings indicate a possible involvement of the selective hydrolysis of α-hydroxy fatty acid-containing sphingomyelin in the process of tumorigenesis in mouse skin.

Key words: Sphingomyelin — α-Hydroxy fatty acid — Ceramide — Initiation-promotion regimen

Sphingomyelin, or ceramide phosphorylcholine, is a ubiquitous membrane component in mammalian tissues and cells, providing a donor group for hydrogen bonding and contributing to the formation of a stable membrane structure. Its reactivity in thymocytes has been found to be altered during blastogenesis by stimulation with a mitogen,[1] and its hydrolysis, catalyzed by cytosolic sphingomyelinase in the so-called sphingomyelin cycle, has been found to occur in association with growth inhibition, induction of differentiation and programmed cell death.[2] Treatment of HL-60 cells with tumor necrosis factor-α,[3] vitamin D_3[4] or interleukin-1[5] induced an acute decrease in the concentration of sphingomyelin with a concurrent increase in the concentration of ceramide,[2–6] and exogenous cell membrane-permeable C_2-ceramide and natural ceramides were found to have antiproliferative and differentiation-inducing effects,[7–10] though conflicting results have been reported regarding the involvement of ceramides in cellular functions.[11] In mammalian skin, a marked increase in ceramide concentration occurs during epidermal differentiation, and ceramides are the major lipid constituent of the stratum corneum[12]; however the mechanism underlying the formation of ceramides is not clearly understood.

In this paper, we report that α-hydroxy fatty acid-containing sphingomyelin (HFA[7]-sphingomyelin), which has not been found in mammalian tissues except for the testis or spermatozoa,[13] is the dominant molecular species in murine epidermis, and that its concentration is reduced in papillomas induced by a standard initiation-promotion regimen in mice.

MATERIALS AND METHODS

Preparation of lipids Epidermal scrapings (approximately 100 mg) were obtained from various murine strains including ICR, Balb/c, Hairless, SENCAR and SSIN mice. Papillomas (approximately 250 mg), induced in the skin of SENCAR and SSIN mice by topical application of 10 nmol of DMBA followed 2 weeks later by twice-weekly topical application of 3.4 nmol of TPA were also obtained. Tumors were harvested at various times after a minimum of 1 week had elapsed since the last TPA treatment. Tissues were lyophilized, and the lipids were extracted with 2 ml aliquots of chloroform/methanol/water (20:10:1, 10:20:1, 20:10:1, and 10:20:1, v/v) at 40°C for 20 min. To remove the acidic lipids, the extracts were combined, applied to a column packed with DEAE-Sephadex A-25 (acetate form, 5 ml bed volume) and eluted with 3 volumes of chloroform/methanol (1:1, v/v) and 1 volume of methanol. The eluted neutral lipids were applied to a TLC plate (Kieselgel 60, Merck, Darmstadt), where the phospholipids were separated by

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[7] Abbreviations: HFA, α-hydroxy fatty acid; DMBA, 7,12-dimethylbenz[*a*]anthracene; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; NFA, nonhydroxy fatty acid.

development with chloroform/methanol/water (65:35:8, v/v, solvent A) and ceramides by development with chloroform/methanol/acetic acid/water (90:7:1:0.7, v/v, solvent B), followed by staining of the lipids with cupric acetate-phosphoric acid reagent. For the detection and quantitative analysis of sphingomyelins, the TLC plate was developed with solvent A and the lipids were stained by the TLC-immunostaining method with monoclonal anti-sphingomyelin antibody VJ-41, as described previously.[1] The spots visualized with H_2O_2 and 4-chloro-1-naphthol were analyzed densitometrically with sphingomyelin purified from human brain as the standard.

Isolation and structure analyses of sphingomyelins Sphingomyelin was purified from the neutral lipid fraction of the normal epidermis of SENCAR mice by column chromatography (Iatrobeads, Iatron Co., Tokyo) with stepwise elution with *n*-hexane/diethylether (4:1) and chloroform/methanol (19:1, 4:1, 1:1 and 1:4). The sphingomyelins eluted with chloroform/methanol (1:1) were visualized as three bands on TLC and were further purified by elution through an Iatrobeads column with a linear gradient of chloroform/methanol/water of 65:35:8 to 5:5:1. Individual bands corresponding to sphingomyelins were analyzed by negative ion FAB-MS (HX-110, JEOL Co., Tokyo)[14] with triethanolamine as a matrix and the fatty acid and long chain base compositions of sphingomyelins were analyzed by GLC and GLC-MS (M-80, Hitachi, Tokyo). After methanolysis with aqueous methanolic HCl at 70°C for 16 h,[15] the fatty acid methyl esters were extracted with *n*-hexane and the long chain bases with diethylether after adjustment of the pH of the lower methanol phase to 11. Prior to the analysis, HFA methyl esters were converted to the trimethylsilyl derivatives and the long chain bases to the *N*-acetyl-*O*-trimethylsilyl derivatives.[16] In addition, the sphingomyelins were treated with phospholipase C (*Cl. welchii*) according to the method of Stahl,[17] and the resultant ceramides were extracted with chloroform/methanol (2:1) and subjected to TLC developed with solvent B.

RESULTS AND DISCUSSION

Sphingomyelins in the epidermis of various murine strains In contrast to human brain sphingomyelins, which give two bands on TLC, with lignoceric acid and palmitic acid as major fatty acids corresponding to the upper and the lower bands, respectively, sphingomyelins in murine epidermis gave three bands on TLC (Fig. 1). The positions of the upper and middle bands were similar to those of the bands corresponding to sphingomyelins in human brain, and the most polar one (SM3), which was more polar than authentic palmitic acid (C16:0)-containing standard sphingomyelin, was the predominant form in the epidermis of all murine strains examined. The three epidermal sphingomyelin bands were stained with monoclonal anti-sphingomyelin antibody as shown in Fig. 1. The sphingomyelins corresponding to each of the bands were purified from the epidermis of SENCAR mice and analyzed by negative ion FAB-MS (Fig. 2). The negative ion FAB-MS spectra of these sphingomyelins showed characteristic ion peaks of $[M-15]^-$ and $[M-86]^-$, depending on whether a methyl group or a choline group was cleaved off, respectively, but the $[M+\text{acetate}-CH_4]^-$ ion peaks reported previously[18] were not observed. The $[M-15]^-$ and $[M-86]^-$ ion peaks for SM1 were observed at m/z 799 and 797, and m/z 728 and 726, corresponding to C24:0- and C24:1-sphingomyelin, respectively, and those for SM2 at m/z 687 and m/z 616, corresponding to C16:0-sphingomyelin. On the other hand, SM3 gave an $[M-15]^-$ ion peak at m/z 703 and an $[M-86]^-$ ion peak at m/z 632, indicating that the major fatty acid of SM3 is α-hydroxypalmitic acid. The product of hydrolysis of SM3 with phospholipase C migrated to a position lower than that of the standard C24h:0-containing ceramides on TLC (Fig. 3). The negative ion FAB-MS of SM3 confirmed that the product of hydrolysis with phospholipase C was a ceramide with α-hydroxypalmitic acid (C16h:0), giving a major molecular ion $[M-H]^-$ peak at m/z 552 (data not shown). In addition, the major fatty acids of SM1, SM2 and SM3 as determined by GLC and GLC-MS analyses were C24:0 and C24:1, C16:0 and C16h:0(α), respectively (Table I). Finally, the long chain base in SM1, SM2 and SM3 was determined to be 4-sphingenine. Thus, α-hydroxypalmitic acid-containing sphingomyelin was shown to be the most abundant molecular species in murine epidermis.

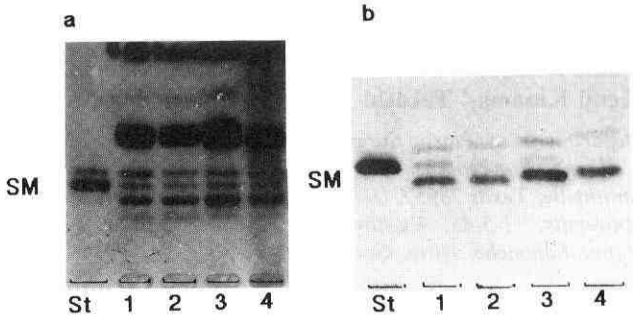

Fig. 1. TLC of sphingomyelins in the epidermis of various murine strains stained with cupric acetate-phosphoric acid reagent (a) and TLC immunostaining with VJ-41(b). Neutral lipids corresponding to 0.5 mg of dry weight were developed with chloroform/methanol/water (65:35:8, v/v). SM, sphingomyelin; St, standard C16:0-sphingomyelin; 1, ICR mouse; 2, Balb/c mouse; 3, Hairless mouse; 4, SENCAR mouse.

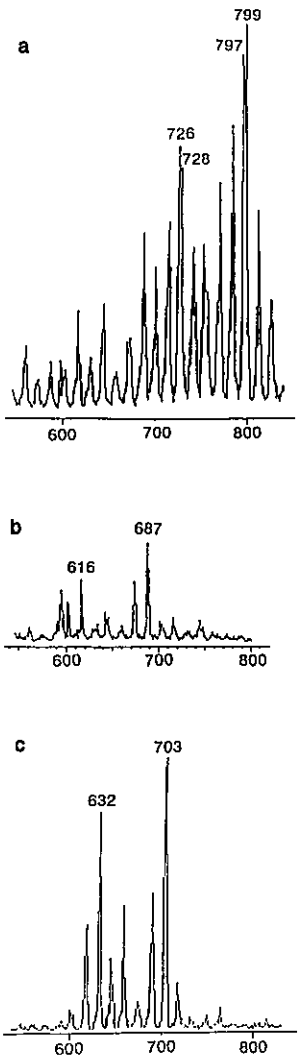

Fig. 2. Negative ion FAB-MS of the sphingomyelins purified from the epidermis of SENCAR mice corresponding to the upper (SM1, a), middle (SM2, b) and lower (SM3, c) TLC bands.

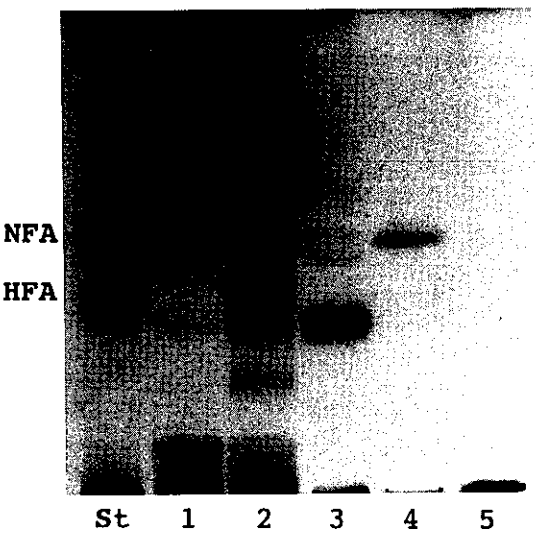

Fig. 3. TLC of ceramides in the epidermis (1) and papillomas (2) of SENCAR mice, and the products of hydrolysis of SM3 purified from the epidermis of SENCAR mice (3), and of sphingomyelin purified from human brain (4) with phospholipase C, intact SM3 (5). Neutral lipids corresponding to 0.5 mg dry weight were developed with chloroform/methanol/acetic acid/water (90:7:1:0.7) and then stained with cupric acetate-phosphoric acid reagent. St, standard ceramides purified from human brain; NFA, C24:0-ceramide; HFA, C24h:0 (α)-ceramide.

Table I. Fatty Acid Compositions of the Upper (SM1), Middle (SM2) and Lower (SM3) Sphingomyelin Purified from the Epidermis of SENCAR Mice

Fatty acid	SM3	SM2	SM1
13h:0	2.0		
14:0	3.2	3.6	3.7
15:0	2.1	1.4	2.3
16:0	10.7	49.8	7.8
16h:0	48.7		
17:0		4.2	2.7
18:0	9.4	13.5	7.6
20:0		1.8	5.9
21:0			1.6
22:0	5.8	11.7	8.5
23:0	2.3		10.1
24:1			15.5
24:0	15.8	3.2	22.7
26:0		10.8	11.6
Total (%)	100.0	100.0	100.0

Molecular alteration of sphingomyelins and ceramides in skin papillomas from SENCAR and SSIN mice As shown in Fig. 4, analysis of lipids in skin tumors demonstrated that the SM3 concentration was negligible in the papilloma from SENCAR mice and markedly reduced in the papilloma from SSIN mice, compared to the control epidermis of both strains. Since the concentrations of SM1 and SM2 in the papillomas were similar to those in the control epidermis (Table II), the SM3 concentration was concluded to be characteristically reduced in the papilloma. In addition, as shown in Figs. 3 and 5, although the concentrations of NFA-containing ceramides were not markedly different between the control epidermis and papilloma, those of HFA-containing ceramides, including HFA-containing phytosphingosines, were dramatically increased in the papilloma (Kitano *et al.*, manuscript in preparation), suggesting that the selective cleavage of

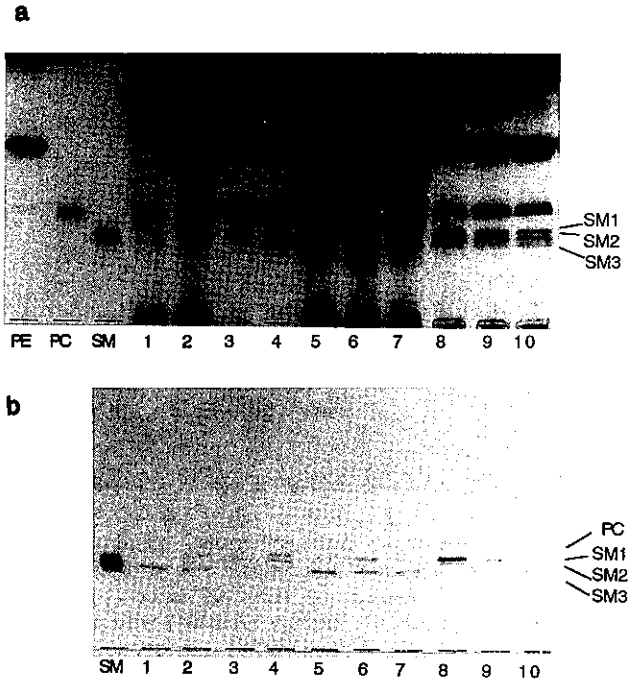

Fig. 4. TLC (a) and TLC-immunostaining (b) of neutral lipids extracted from the epidermis (1, 2) and papillomas (3, 4) of SSIN, and from the epidermis (5–7) and papillomas (8–10) of SENCAR mice. Neutral lipids corresponding to 0.5 mg dry weight were developed with chloroform/methanol/water (65:35:8) and were visualized by staining with cupric acetate-phosphoric acid reagent and by immunostaining with monoclonal anti-sphingomyelin antibody VJ-41 (b), which crossreacted with disaturated fatty acid-containing phosphatidylcholine (PC), but not with unsaturated fatty acid-containing PC as reported previously.[1] PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin.

HFA-containing sphingomyelin contributes to the increased concentration of HFA-containing ceramides as a molecular event characteristic of the papilloma. In fact, the hypertrophic lesions induced by treatment with TPA alone exhibited no significant alteration in the concentrations of HFA-sphingomyelin and HFA-ceramide (data not shown). Although it has not been clarified whether α-hydroxy long chain fatty acid- and phytosphingosine-containing ceramides (hydroxylated ceramides) were derived from cleavage of glycosphingolipids or *de novo* synthesis, large amounts of the hydroxylated ceramides which were not utilized were accumulated in papillomas, suggesting that the anabolic enzyme CDP-choline: ce-

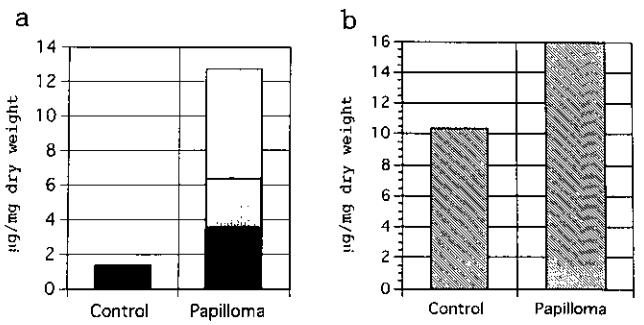

Fig. 5. Concentrations of ceramides with HFA-ceramides (a) and NFA-ceramides (b) in the normal epidermis (control) and papilloma of SENCAR mice. Open column, α-hydroxy long chain fatty acid (C20h:0-C26h:0)-containing ceramides; dotted column, α-hydroxy fatty acyl phytosphingosine; closed column, α-hydroxy short chain fatty acid (C14h:0-C18h:0)-containing ceramides.

Table II. Concentrations of the Upper (SM1), Middle (SM2) and Lower (SM3) Sphingomyelin in the Epidermis of Various Murine Strains and the Papillomas (μg/mg of dry weight)

		SM1	SM2	SM3
SSIN	Control	0.5±0.2 (31.3)	0.1±0.0 (6.3)	1.0±0.1 (62.4)
	Papillomas	0.6±0.2 (37.5)	0.7±0.1 (43.8)	0.3±0.0 (18.7)
SENCAR	Control	0.4±0.1 (25.0)	0.1±0.0 (6.3)	1.1±0.1 (68.7)
	Papillomas	0.6±0.1 (46.0)	0.5±0.1 (38.5)	0.2±0.0 (15.3)
ICR		0.2 (14.3)	0.2 (14.3)	1.0 (71.4)
Balb/c		0.2 (18.2)	0.1 (9.1)	0.8 (72.2)
Hairless		0.2 (11.8)	0.1 (5.9)	1.4 (82.3)

Percentages of SM1, SM2 and SM3 in the total sphingomyelin are shown in parenthesis.

ramide cholinephosphotransferase recognizes the ceramide moiety. To clarify the metabolic processes occurring in papillomas, analysis of the specificity of catabolic and anabolic enzymes for sphingomyelin is required and is in progress in our laboratory. In addition, in the process of epidermal differentiation as well as programmed death of keratinocytes, formation of ceramides through the sphingomyelin cycle may play an important role in triggering the ceramide-mediated signal transduction pathways,[19] and disordered differentiation induced by exposure to chemical carcinogen and promoter might provide a useful model for investigating the functional significance of the lipid mediators in these processes.

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