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ACTIVATION OF KERATINIZATION OF KERATINOCYTES FROM FETAL RAT SKIN WITH N-(O-LINOLEOYL) ω-HYDROXY FATTY ACYL SPHINGOSYL GLUCOSE (LIPOKERATINOGENOSIDE) AS A MARKER OF EPIDERMIS [1]

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SUMMARY: To elucidate the functional significance of sphingolipids altered in the epidermal differentiation, we examined the effects of sphingolipids on the activity of transglutaminase and the formation of cornified envelopes in the keratinocytes from fetal rat skin. N-(O-linoleoyl) ω-hydroxy fatty acyl sphingosyl glucose (lipokeratinogenoside) that was characteristically contained in the mammalian epidermis, as well as nonhydroxy fatty acid-containing GalCer and GlcCer, significantly enhanced the activity and the formation, but no or rather inhibited activity was observed with ceramides, GalCer with α-hydroxy fatty acid, saponified lipokeratinogenoside, etc. This indicates that skin-characteristic lipokeratinogenoside functions to regulate the transglutaminase for the formation of cornified envelopes in the process of keratinization.

In many visceral organs, glycosphingolipids are rather minor components in contrast to phospholipids and cholesterol. However, particular attention has been paid to the biological activities of glycosphingolipids, for example, as differentiation-inducer(3,4), proliferation-regulator(5), receptor(6) and recognition molecules(7). Furthermore, it is known that not only glycosphingolipids, but also their constituents such as sphingosine and ceramides, participate in regulation of C-kinase activity(8) and erythroblast formation(9),

[1] The glycolipids nomenclature and symbols are used according to an IUPAC-IUB document (1) and Svennerholm's system (2).

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respectively. As clearly shown in our previous paper(10), the mammalian epidermis is quite a unique tissue in its significantly high concentration of glycosphingolipids, which are thought to be closely related to the physical barrier, viscoelasticity and intercellular adhesion of skin(9,10). In comparing the lipid composition of guinea pig epidermis with that of dermis, N-(O-linoleoyl) ω-hydroxy fatty acyl sphingosyl glucose (lipokeratinogenoside) was found to be characteristically contained in the epidermis and to make up 44.0% (footpad skin) and 16.7% (dorsal skin) of the total monohexosyl sphingolipid fraction. It should be newly synthesized during the differentiation process of epidermis including loss of nuclei and keratinization, and is thought to be related to the differentiation process as described above. To elucidate the possible involvement of lipokeratinogenoside in the differentiation of the epidermis, we examined the effects of exogenous ceramides, psychosine, GalCer, GlcCer, lipokeratinogenoside, LacCer, I^3SO_3-GalCer and II^3NeuAc-Gg_4Cer on the differentiation of keratinocytes, which were established from fetal rat skin.

MATERIALS AND METHODS

Materials : Ceramides were prepared from bovine brain sphingomyelin by phospholipase C digestion(13). The isolation of GalCer with nonhydroxy fatty acids (NFA-GalCer) and with α-hydroxy fatty acids (HFA-GalCer) , I^3SO_3-GalCer, LacCer and II^3NeuAc-Gg_4Cer from bovine brain was carried out according to the method reported previously (14). GlcCer which is mixture of NFA-GlcCer and HFA-GlcCer (62:38) was isolated from bovine spleen by similar procedure. Lipokeratinogenoside fraction was prepared from guinea pig epidermis as discribed previously (8). From the fraction a sample used in the present experiments was obtained. This sample was found to contain N-(O-linoleoyl) ω-hydroxy dotriacontamonoenoyl sphingosyl glucose (64.2%) as a major compound besides other lipokeratinogenosides, N-(O-linoleoyl) ω-hydroxy tetratriacontamonoenoyl sphingosyl glucose (28.7%) and N-(O-linoleoyl) ω-hydroxy tritriacontamonoenoyl sphingosyl glucose (7.1%) (unpublished observation). Psychosine

and saponified lipokeratinogenoside from GalCer and lipokeratinogenoside, respectively, were prepared by alkaline treatment(15, 16). 12-O-tetradecanoyl phorbol 13-acetate and linoleic acid were purchased from Sigma (St. Louis, MO).

Cell culture : FRSK cells, which were established from fetal rat skin by Dr. K. Indo (Hyogo Medical College, Kobe, Japan)(17) were provided through The Cell Bank of the Japanese Cancer Research Association. Cells were cultured in Eagle's minimum essential medium supplemented with kanamycin (60mg/ml) and 10% fetal calf serum at 37°C in 5% CO_2 in air.

Effects of exogenous ceramides, GalCer and lipokeratinogenoside on transglutaminase activity of FRSK cells : Cells were plated at a density of 1 x 10^6/60mm in a plastic dish and cultured for 18 hrs. Then the medium was changed to that containing various concentrations of ceramides, GalCer or lipokeratinogenoside, which were dissolved in ethanol and mixed with the medium in the final ethanol concentration of 1%(v/v). After cultivation for 48 hrs, the cells were washed once with PBS, harvested with a rubber policeman and homogenized with 100mM Tris-HCl buffer, pH 7.5, containing 2mM EDTA, 20mM $CaCl_2$ and 4mM dithiothreitol. Transglutaminase activity in the homogenates was measured by the method described by Lorand and Gotoh (18).

Effects of exogenous ceramides, lipokeratinogenoside and several glycosphingolipids on cornified envelope formation of FRSK cells : FRSK cells cultured in the medium containing ceramides or other glycosphingolipids for 96 hrs were washed with PBS(-) containing 0.02% EDTA and harvested by treatment with 0.25% trypsin in PBS. The number of cornified envelopes was determined according to the method of Sun and Green (19).

RESULTS

Effects of exogenous ceramides, GalCer and lipokeratinogenoside on transglutaminase activity of FRSK cells

Transglutaminase is a marker enzyme for the keratinization process and is known to be significantly activated with 12-O-tetradecanoyl phorbol-13-acetate (TPA) to enhance the process. In fact, cultivation of FRSK cells in the presence of TPA(0.1μg/ml) for 18 hrs gave a 245%(SD±29%) increase of the activity in the cells cultured without TPA. As shown in Fig.1, although ceramides and HFA-GalCer did not enhance the activity at the various

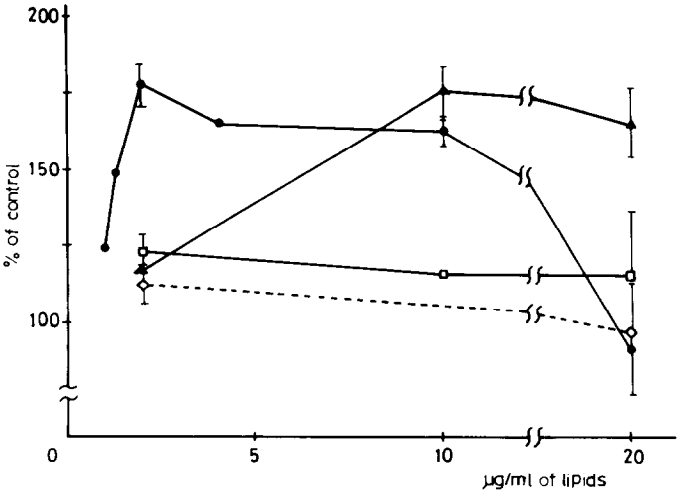

Fig.1. Effects of exogenous ceramides, GalCer and lipokeratinogenoside on the activity of transglutaminase in FRSK cells. The enzyme activity in the cell cultures with sphingolipids at the concentration indicated in Fig. 1 for 18 hrs was compared with that of cultures without sphingolipids (control). Each point is the mean of three separate experiments. ●, lipokeratinogenoside; ▲, nonhydroxy fatty acid-containing GalCer; □, α-hydroxy fatty acid-containing GalCer; ◇, ceramides; I, SD.

concentrations tested, lipokeratinogenoside at 1 to 10μg/ml and NFA-GalCer at 10 to 20μg/ml activated the enzyme up to 173% of the control. Since the fatty acid composition of ceramides prepared from bovine brain sphingomyelin was similar to that of NFA-GalCer, the carbohydrate moiety of NFA-GalCer was thought to be essential for the activation of the enzyme. In addition, removal of the esterified fatty acid from lipokeratinogenoside diminished the activity.

Effects of exogenous ceramides, lipokeratinogenoside and several glycosphingolipids on cornified envelope formation of FRSK cells

As a result of activation of transglutaminase, cornified envelopes, which are mainly constructed by cross-linking of keratin, are formed around the cells. After cultivation for 96 hrs, the number of the cells with cornified envelopes was counted and compared with that of the cells cultured in the medium. As shown

Table I. Effects of exogenous ceramides and various glycosphingolipids on the formation of cornified envelopes in FRSK cells

Addition		% of control
no addition		100
ceramides	10μg/ml	105
psychosine	10	108
NFA-GalCer	10	173
HFA-GalCer	10	67
GlcCer	10	149
lipokeratinogenoside	2	132
	10	156
saponified-lipokeratinogenoside	10	76
LacCer	10	93
I^3SO_3-GalCer	10	86
II^3NeuAc-Gg_4Cer	10	82
TPA	0.1	180

in Table I, TPA at the concentration of 0.1μg/ml significantly activated the formation of cornified envelopes. Similarly, NFA-GalCer, NFA-GlcCer and lipokeratinogenoside, but neither ceramides, HFA-GalCer, linoleic acid, nor saponified lipokeratinogenoside, enhanced the formation of cornified envelopes in accordance with the effects of NFA-GalCer and lipokeratinogenoside on transglutaminase. HFA-GalCer containing α-hydroxy fatty acids and saponified lipokeratinogenoside containing ω-hydroxy fatty acids rather diminished the formation of cornified envelopes. Thus α- or ω-hydroxy group in the ceramide moiety inhibited the activity. In addition, from the result that addition of psychosine, LacCer, I^3SO_3-GalCer and II^3NeuAc-Gg_4Cer did not enhance the formation of cornified envelopes, the ceramide residue without hydroxy fatty acid, as well as the monohexosyl moiety without any modification were required for the activity. But, the structure of lipokeratinogenoside was found to be effective to reduce the optimum dose.

DISCUSSION

Differentiation-associated modification of glycosphingolipids is frequently observed in mammalian cells, but the mode of

modification usually differs depending on the cell types originating from different animal species. Also glycosphingolipids newly appearing as a consequence of the differentiation have recently been recognized to be involved in triggering or directing the process of differentiation. In the differentiation of keratinocytes from the basal to the cornified layers, a characteristic gradient of the sphingolipid contents and composition was observed among several mammals, as reported previously(20,21,22). Monohexosyl ceramides and lipokeratinogenoside were increased in concentration from the basal to the granular layers, and the content of ceramides was increased from the granular to the cornified layers probably due to the enzymatic cleavage of monohexosyl ceramides, lipokeratinogenoside or sphingomyelin. Among the sphingolipids altered during the differentiation of keratinocytes to form the epidermis of skin, lipokeratinogenoside was noticeable because of the molecule detectable only in the skin, suggesting that it is significantly involved in the process of keratinization. In fact, the transglutaminase which functions as the cross-linking enzyme of keratin to form the cornified envelopes. Therefore, the synthesis of skin-characteristic lipokeratinogenoside is thought to significantly regulate the activity of a key enzyme for the process of keratinization.

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