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Phenotypic changes in 3T3 cells associated with the change of sphingolipid synthesis by a ceramide analog, 2-decanoylamino-3-morpholino-1-phenylpropanol (compound RV538)

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A culture of BALB/c 3T3 cells grown in the presence of 40 μM of the ceramide analog compound RV538 (2-decanoylamino-3-morpholino-1-phenylpropanol) for several passages caused a substantial decrease in the level of all glycosphingolipids and an accumulation of ceramide and sphingomyelin. Associated with these chemical changes of sphingolipid composition and metabolism, the following phenotypic changes were observed: (i) loss of the cobblestone appearance at cell density saturation and development of fibroblastic appearance with partial overlapping of cells; (ii) reduction of cell growth rate; (iii) enhanced production of lactic acid; (iv) enhanced rate of glucose transport; and (v) higher incidence of large colony formation with infiltrating appearance in soft agar. Cell morphology changes, lactate pruduction, and enhanced sugar uptake were reversed by co-culturing cells with gangliosides, particularly trisialogangliosides. Thus, these phenotypic changes mimicking those of oncogenically transformed cells are closely related to the blocked synthesis of glycolipids in these cells, whereas other changes may be caused by an accumulation of ceramide and sphingomyelin.

Ceramide analog inhibitor; Glycosphingolipid; Phenotype; Cell growth

1. INTRODUCTION

Glycosphingolipids in cell membranes have been implicated as regulators of cell growth and mediators of cell social interaction [1]. As a test of

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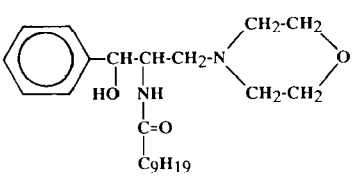
Abbreviations: DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GT, trisialogangliosides of bovine brain (mainly GT_{1b}); TLC, thin-layer chromatography

RV538



Ceramide

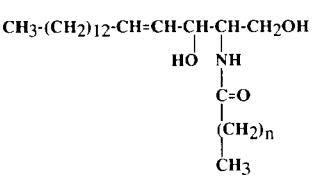


Fig.1. The structure of 2-decanoylamino-3-morpholino-1-phenylpropanol (compound RV538) and ceramide. The compound was crystallized as the hydrochloride (M_r 427).

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this concept, we tried to inhibit synthesis of total glycolipids by application of a ceramide analog, compounds RV538 (2-decanoylamino-3-morpholino-1-phenylpropanol, fig.1), which inhibits UDP-glucose:ceramide glucosyltransferase [2]. Culturing 3T3 cells in the presence of RV538 resulted in a reduced growth rate, but caused remarkable changes of cellular phenotypes similar to those observed in oncogenically transformed cells, i.e., changes in morphology, enhanced production of lactic acid, changes in cell growth behavior in soft agar, and enhanced sugar transport. These RV538-induced phenotypic changes are reported in this paper.

2. MATERIALS AND METHODS

All glycolipids were prepared in this laboratory. The ceramide analog RV538 was synthesized as previously described [2]. [^{3}H]Galactose and [^{3}H]palmitic acid were from New England Nuclear (Boston, MA). Sphingomyelin and Oil Red O were from Sigma (St. Louis, MO). Ceramide was prepared by Smith degradation of cerebroside [3]. Conditions of cell growth in the absence or presence of RV538, and the monitoring of sphingolipid biosynthesis are described in the legend of fig.2. Levels of lactic acid and pyruvic acid in the medium were determined by spectrometry of NAD reduction as described previously [4], using a Sigma kit (Sigma). Glucose transport was determined as described by Christopher et al. [5]. Conditions for culturing cells for the determination of lactic acid and sugar transport are described in the footnote of table 1. Cell growth behavior in soft agar was determined as described in the legend

CPM of each sphingolipid component

	control	RV538-treated
I		
CMH	3160	670
CDH	500	120
GM_3	2070	410
GM_2	580	180
GM_{1a}	860	360
GD_{1a}	1180	400
III		
sphingomyelin	190,000	740,000
ceramide	450	1430

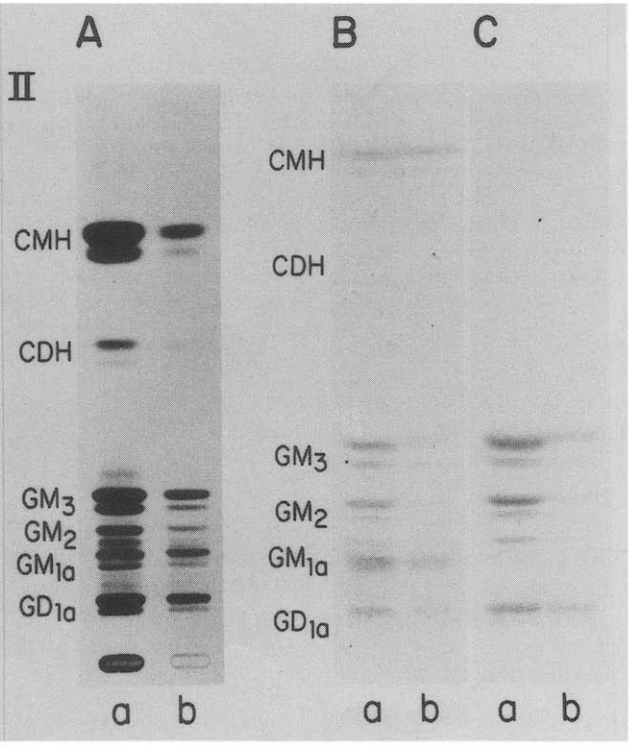

Fig.2. Sphingolipids of control and RV538-treated 3T3 cells. Subconfluent cell cultures grown in DME with 10% FCS were continuously cultured in a new medium containing 3 μCi [^{3}H]Gal/ml in the presence or absence of 40 μM of RV538 for 24 h. Glycolipids extracted from 10^6 cells were purified as acetate, placed in each lane after deacetylation, and developed in chloroform/methanol/water (60:35:8, v/v). Bands corresponding to each glycolipid were scraped and radioactivities were counted (panel I). Each number represents the mean value of three separate experiments. TLC patterns are shown in panel II. Panels show the autoradiogram (A), orcinol staining (B), and resorcinol staining (C) of the plate. (a) Control 3T3 cells; (b) RV538-treated 3T3 cells. Cells cultured in the presence or absence of 40 μM RV538 for 10 passages were metabolically labeled with 10 μCi [^{3}H]palmitic acid/ml for 12 h in the presence or absence of RV538. Extracted cells were Folch partitioned, and the lower phase was washed 5 times with theoretical upper phase. Aliquots of washed lower phase were placed on an HPTLC plate (Merck, Darmstadt, FRG) with ceramide or sphingomyelin standard, developed, and visualized by Dittmer-Lester's spray and hypochlorite-benzidine as described in section 2. The bands corresponding to standard lipids were scraped and counted in a liquid scintillation counter (panel III). Values represent means of three separate experiments.

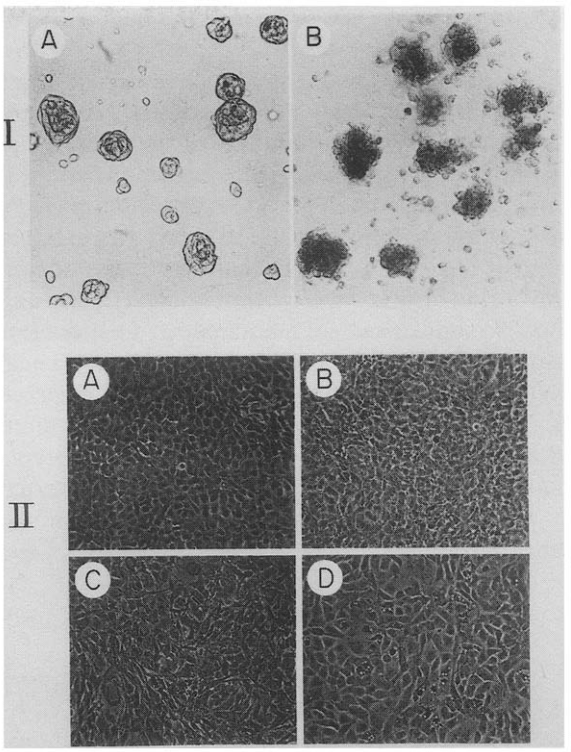

Fig.3. Changes in growth behavior of 3T3 cells induced by RV538 in soft agar or on cell culture plates. (Panel I) Cells cultured in DME with 10% FCS without (A) or with (B) 40 μM RV538 for 10 passages. Cells were trypsinized and suspended in 0.3% agar and overlaid on solid 0.5% agar. Pictures were taken 12 days after plating. (Panel II) Control or RV538-treated cells supplemented after attachment with: (A) no additives (control); (B) 50 μg GT/ml; (C) 40 μM RV538; (D) GT plus RV538. Pictures of confluent cells were taken after several days.

of fig.3. Glycolipids were separated as acetates [6] and analyzed on HPTLC plates. Ceramide was separated from glycolipids and sphingomyelin in dichloroethane/methanol/water (90:20:0.5, v/v), and sphingomyelin was separated from other phospholipids in isopropanol/water (7:3, v/v). Sphingolipids on TLC were visualized by hypochlorite-benzidine [7], and sphingomyelin and other phospholipids were detected by Dittmer and Lester's spray reagent [8].

3. RESULTS

3.1. *RV538-induced changes in sphingolipid synthesis and cell morphology*

The quantities and pattern of biosynthesis of sphingolipids were greatly reduced in 3T3 cells cultured in the presence of 40 μM RV538 for five passages, while accumulation of sphingomyelin and ceramide was greatly increased (fig.2, panels I and III). Associated with these major changes in sphingolipid synthesis, the typical cobblestone appearance of the saturated cell population of BALB/c 3T3 cells was lost, although the cell saturation density was reduced from 2.4 ± 0.2 × 10^5/cm^2 to 1.7 ± 0.05 × 10^5/cm^2.

Table 1

Effect of RV538 and GT on lactic acid production and glucose uptake in BALB/c 3T3 cells

	Untreated	RV538 treated	RV538 and GT treated	GT treated
Lactic acid produced[a] (nmol/10^5 cells per h)	35.3 ± 5.6 (0.6 ± 0.1)	91.4 ± 7.7 (0.3 ± 0.1)	44.7 ± 7.3	35.0 ± 5.2
Glucose uptake[b] (cpm × 10^{-4}/mg protein per 10 min)	8.9 ± 0.1	13.5 ± 0.5	11.0 ± 0.1	4.8 ± 0.1

[a] Cells were cultured for 10 passages in DME containing 10% FCS and 40 μM RV538, and subcultured in new medium in the presence or absence of 40 μM RV538 and/or 50 μg GT per ml. After confluency (6 h), the medium was collected and deproteinated with 8% perchloric acid, and lactic acid content was determined by spectrophotometry. Pyruvic acid was essentially absent in the medium (values in parentheses)

[b] Cells cultured as described above were plated in 35 mm dishes and continuously cultured in the presence or absence of RV538 and/or GT. Adherent cells were incubated for 10 min in DME without glucose or FCS, but containing [^{3}H]deoxyglucose (2 μCi/μg deoxyglucose per ml). After washing with DME without glucose or FCS, cells were solubilized in 0.1 N NaOH/0.1% SDS, and radioactivity and protein content were determined

3.2. *Anomalous phenotypic characteristics of RV538-treated cells*

3T3 cells cultured in a medium containing RV538 displayed various characteristic phenotypic changes: (i) enhanced production of lactic acid, causing the culture medium to become acidic; (ii) enhanced transport of [^{3}H]deoxyglucose (table 1); (iii) anomalous growth in soft agar, i.e. a larger number of colonies with increasing number of cells and an infiltrating diffuse boundary (fig.3); and (iv) accumulation of anomalous perinuclear granules (stained intensely by Oil Red O), which became obvious after 4–5 passages in the presence of RV538. Control cells grown in a medium without RV538 did not display these phenotypic changes, and phenotypes of altered cells reverted to normal when RV538 was removed from the culture medium.

3.3. *Reversible effect of ganglioside on RV538-treated cells*

When 3T3 cells were co-cultured in the presence of RV538 and trisialoganglioside (GT), cell morphology returned to the cobblestone appearance (fig.3), concentration of lactic acid decreased significantly with increasing GT ganglioside concentration (fig.4), and glucose uptake was reduced (see table 1).

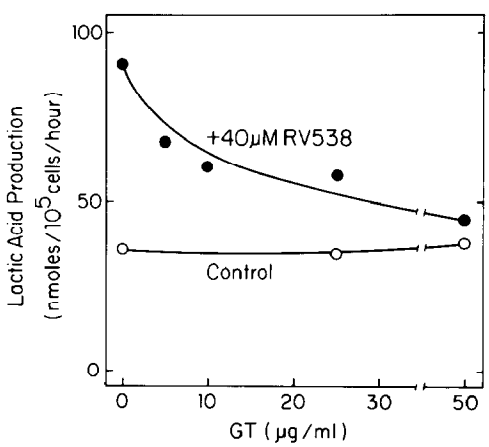

Fig.4. Inhibitory effect of GT on RV538-induced lactic acid production. 3T3 cells were cultured in the presence (●) or absence (○) of 40 μM RV538 with various amounts of GT, as indicated on the abscissa. After confluency, the medium was changed to one with or without RV538 and/or GT, collected 6 h later, and used for determination of lactic acid.

4. DISCUSSION

Although glycolipid alteration associated with differentiation, development, and oncogenesis has been well established [1], a major question remains as to whether the alteration is the cause or the result of phenotypic changes. To answer this question, glycolipids were added to the culture media in various studies [9–12]. Another useful approach is the inhibition of glycolipid synthesis by competitive substrate inhibitors and observation of any changes in cell phenotype; however, this approach has not been well developed. Yunnam and Radin [2] synthesized analogs of ceramides that inhibit the activity of UDP-Glc:ceramide glucosyltransferase, the enzyme that initiates synthesis of essentially all glycosphingolipids except galactosylceramide and derived analogs. The present study shows that in 3T3 cells cultured in the presence of one such analog, RV538 (fig.1), production of all glycolipids (including GlcCer, LacCer, GM_3, GM_2, GM_1, and GD_{1a}) is greatly reduced. Treated cells, however, accumulate several-fold more sphingomyelin and ceramide than do untreated cells. Thus, the synthetic pathway of sphingolipids in treated cells is diverted from the glycosphingolipid to the sphingomyelin pathway.

Associated with such dramatic changes in sphingolipid synthesis induced by RV538, various phenotypic changes (e.g., reduced growth rate, morphological changes, enhanced glucose transport, enhanced lactic acid production, changes in soft agar growth behavior, and appearance of perinuclear lipophilic granules) have been observed. However, it is not clear from the present study which changes are caused by reduction of glycolipid synthesis and which are related to accumulation of ceramide and sphingomyelin.

Accumulation of perinuclear lipophilic granules may coincide with accumulation of sphingomyelin and ceramide. Interestingly, Lipsky and Pagano [13] observed accumulation of a fluorescent ceramide analog (*N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]aminocaproyl-sphingosine in mitochondrial membranes added exogenously to fibroblasts. It is possible that enhanced lactic acid production could reflect impaired oxidative phosphorylation in mitochondria due to accumulation of ceramide in the mitochondrial membrane. Further study is necessary to clarify the possible role of ceramide

and sphingomyelin in mitochondrial function. Reduction of cell growth may result from low efficiency of ATP production caused by impaired mitochondrial function. On the other hand, loss of the cobblestone appearance and other morphological changes may be related to reduction of glycolipid synthesis, although a definite functional correlation remains to be established.

Interestingly, co-culturing of cells with various gangliosides, particularly GT, was observed to reverse the phenotypic changes caused by RV538, e.g., it caused reduction in lactic acid production and sugar transport, and reversion of morphological changes. These observations suggest that these phenotypic changes are partially related to the reduction of ganglioside levels, and that approaches involving the modification of sphingolipid metabolism by ceramide analog may offer a useful tool for studies of sphingolipid function in general.

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