J. Appl. Cosmetol. **10**, 13-16 (January - March 1992)

EFFECT OF SOME EXOGENOUS GLYCOSPHINGOLIPIDS ON HUMAN KERATINOCYTES IN CULTURE

I. Varani, A. Terzaghi, L. Donati*, M. Marazzi*, S. Garbin*, G. Tettamanti and M. Masserini

*Dipartimento di Chimica e Biochimica Medica e * Istituto di Chirurgia Plastica e Ricostruttiva, Università di Milano, Via Saldini 50, 20133 Milano, Italy.*

Received: October 30, 1991

Key words: *Cell Cultures, Keratinocytes, Glycosphingolipids, Proliferation*

Synopsis

We have studied the influence of different glycosphingolipids on human keratinocytes in culture and their action on cellular proliferation. We found none of the molecules utilized had any cytotoxic effect. Moreover, the glycosphingolipids could modify the proliferation rate of keratinocytes. These molecules could play a role as epidermal eutrophic, repairing and protective agents and could also be used as vehicles for cosmetically active products.

Riassunto

Prove condotte utilizzando diversi glicosfingolipidi hanno dimostrato che queste molecole non hanno effetto citotossico sulle colture in vitro di cheratinociti umani, e che alcuni sono in grado di modificare la capacità proliferativa di queste cellule. Essi potrebbero quindi essere utilizzati come agenti eutrofici, riparativi e protettivi dell'epidermide ed anche come veicoli per sostanze cosmeticamente attive.

Introduction

Glycosphingolipids are amphipatic molecules, which are normal components of cellular membranes (1, 2), and are also present in the plasma membranes of epidermal cells. When they are exogenously added to "in vitro" cultured cells, they become strongly incorporated in the plasma membrane (3,4). In this situation, they become biochemically identical to endogenous glycolipids and mimic their metabolic pathway. Moreover, the exogenous enrichment of cells can cause a modified architecture of the cellular membranes, and offers the possibility to modify the cellular response. In the present work, we begin to study if the exogenous treatment with these molecules might play a role in epidermal structure and function.

Materials and methods

All the materials for cell culture were from Irvine Scientific (Santa Ana, CA, U.S.A.). Mitomycin C, insulin, transferrin, triiodothyronine, adenine and cholera toxin were from Sigma Chem. Co. (St. Louis, MO, USA). 3H-thymidine ([3H]TdR) from Amersham (Little Chalfont, U.K.). Glycosphingolipids were extracted and purified from beef brain. Their purity was over 99%.

Preparation of epidermal cell suspension

The epidermal cell suspension was prepared by enzymatic release of cells from the epidermis after initial separation from the dermis. The skin ($2cm^2$ area) was cut with surgical scissor to small slices. These were treated in Trypsin/EDTA (Trypsin 0.5g (1:250)/EDTA 0.2g; 1h at 37°C. The dermis was discarded and the epidermal cells were harvested and then centrifuged. The pellet was resuspended in medium without EGF and seeded in 25 cm^2 culture flasks with a layer of mitomycin C-treated 3T3 fibroblasts.

3T3 fibroblasts culture

Murine 3T3 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% foetal calf serum (FCS). Confluent cultures of 3T3 cells were treated with mitomycin C (4μg/ml) (5,6) for 2h, washed twice with PBS and harvested with 0.2% EDTA in PBS to detach the cells from the culture flask. The cells were plated in flasks for human keratinocyte culture with a density of 20000 cells/cm^2, 4 h prior to initiation of epidermal cell cultures.

Epidermal cell culture

Human ECs were cultured as described by Rheinwald & Green (7). The cells were inoculated at a density of 8000 cells/cm^2 into 25 cm^2 flasks containing the mitomycin C- treated 3T3 fibroblasts. With this density the keratinocytes, in normal condition, reach a confluent layer in 8-9 days. The growth medium (10ml) consisted of a 3:1 mixture of DMEM and Ham's F12 medium supplemented with 10% FCS, 5μg/ml insulin, 5μg/ml transferrin, $2x10^{-9}$ M triiodothyronine, $1.8x10^{-4}$ M adenine and 1nM cholera toxin. 48 h after initiation and subsequently every second day, the cultures were fed with the same medium containing 10μg/ml epidermal growth factor (EGF). For the harvesting of different glycolipid cultures ten plates (35mm culture dishes) were prepared in all experiments.

Treatment with glycosphingolipids

Glycosphingolipids (GM1, asialoGM1, glucosylceramide, galactosylceramide, lactosylceramide and sulfatide) were dissolved in the culture ECs medium with EGF and without FCS. The medium containing a given glycosphingolipid (10^{-5}M), was added to the cell cultures every day for a period of 1h. After incubation, the medium was removed and the cells were washed twice with 1ml PBS. The ECs medium was again added, except to the cells for thymidine incorporation.

Thymidine incorporation assay

The proliferative activity of the different glycolipids was assessed by monitoring [^{3}H] thymidine ([^{3}H] TdR) incorporation. The [^{3}H] TdR was dissolved (4μCi/ml) in ECs medium supplemented with 10% FCS. At different times, immediately after the treatment with glycolipids, the cultures were pulsed with 4μCi/plate [^{3}H] TdR (Amersham International) and cultured for an additional 6 h. The cells were washed twice with 1ml PBS. The cells were treated overnight in 300μl of 0.5 M NaOH, and 10μl of the solution were counted for radioactivity with a Packard ß- counter.

Results

Microscopic examination showed a normal growth of human keratinocytes treated or not treated with glycosphingolipids. 4 - 5 days after the epidermal cells were seeded, colonies were present in all culture plates. The morphology of untreated and treated cells was the same. Nine days after the inoculation, the cells formed a confluent monolayer, as predicted. The [^{3}H] TdR incorporation was measured after one day (T0), 2 days (T1), 5 days (T2) and 8 days (T3). After 8 days, we observed a marked decrease of [^{3}H] TdR incorporation in both the glycosphingolipid-treated cultures and the control ones. In these conditions the cells become confluent and this explains the low values of [^{3}H] TdR incorporation obtained in all samples.

The % of [^{3}H] TdR incorporation at confluence is 5% of that observed after 1 day in culture. A different behaviour was observed between keratinocytes which had been treated with different glycosphingolipids. The keratinocytes present in the untreated cultures showed a decrease of [^{3}H] TdR incorporation for all the experiment (Fig. 1). We observed that the cells treated with GM1 and asialoGM1 showed at T0 a decrease (-28% for both) of [^{3}H] TdR incorporation in respect to the control. In T1 and T2, there was an increase of [^{3}H] TdR incorporation (T1: GM1 + 23%, asialoGM1 + 26%: T2: GM1 + 65%, asialoGM1 + 56%) (Fig. 2). The epidermal cells treated with the two glycolipids showed a strong decrease of incorporation at T3, that anyway remained higher than untreated cells (+45% and + 31% respectively).

With glucosylceramide (fig. 3), at T0 and T1, values of [^{3}H] TdR incorporation were similar to those obtained for the control (-13% and -3.6% respectively). After 5 days of treatment with the glycolipid (T2), the [^{3}H] TdR incorporation reached the values observed with GM1 and asialoGM1 (+64.4%).

Lactosylceramide and sulfatide (fig. 3) produced at T0, at T1 and at T3, [^{3}H] TdR incorporation levels comparable with the control cells. An increase was observed at T2 (+35% and +45%). With galactosylceramide the values of radioactivity incorporation were: T0: -28%, T1: -18%, T2: -10.5%. At T3 a small increase of incorporatiou (+26%) was found compared to the control.

Discussion

Microscopic examination and [^{3}H] TdR incorporation indicated a normal proliferation for cultures treated with all glycolipids. From this point of view glycosphingolipids do not interfere with the epidermal cell cultures. However, they affect the proliferation rate of keratinocytes; the results obtained with [^{3}H] TdR incorporation showed that different glycosphingolipids affect the cell cultures. GM1 and asialoGM1 efficiently promoted the rate of cellular proliferation for the whole duration of the experiment, while galactosylceramide caused a decrease of the rate.

The other glycosphingolipids (glucosylceramide, lactosylceramide and sulfatide) positively affected the proliferation as shown by the experiments. In conclusion, some glycosphingolipids seem to exert a positive effect on keratinocytes proliferation. This eutrophic effect could be employed for cosmetic purposes on human skin.

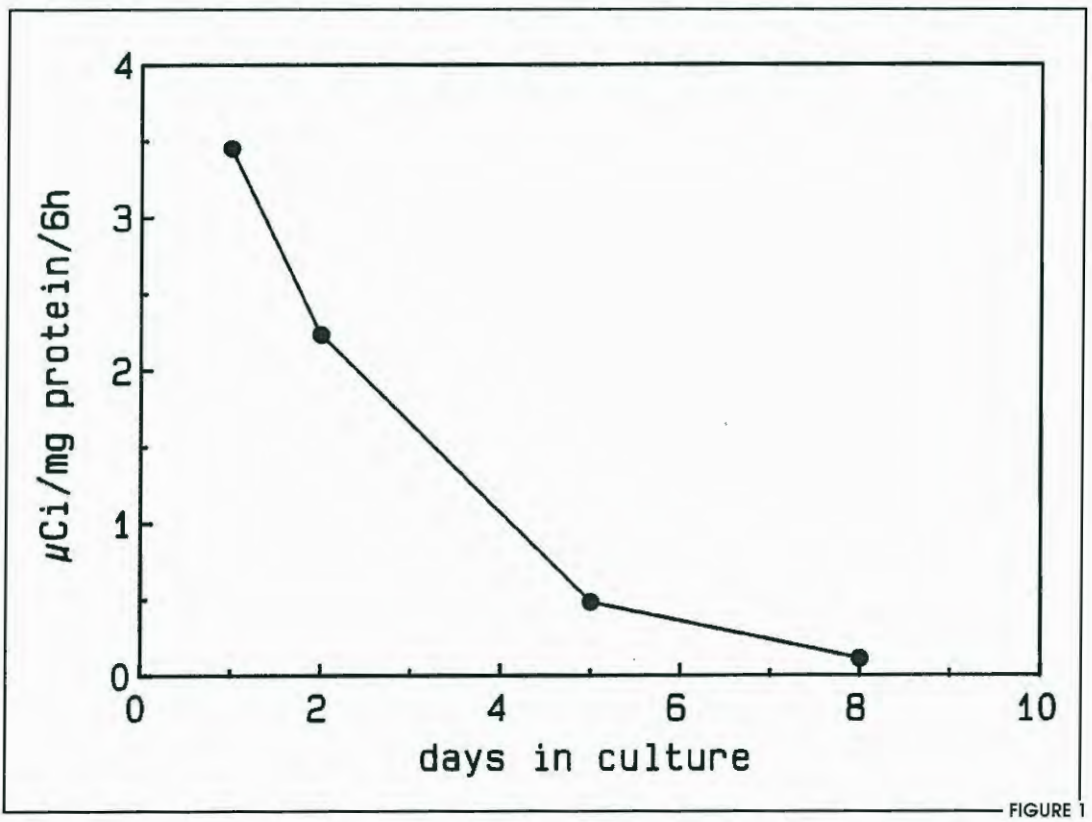

FIGURE 1

3H-thymidine incorporation into human keratinocytes at different times in culture.

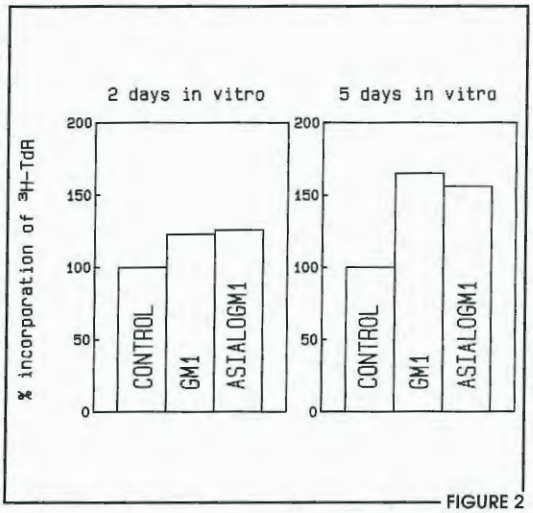

FIGURE 2

³H-thymidine ([³H] TdR) incorporation after treatment of human keratinocytes in culture with different glycolipids. [³H] TdR incorporation into keratinocytes not treated with glycolipids taken as 100% (control).

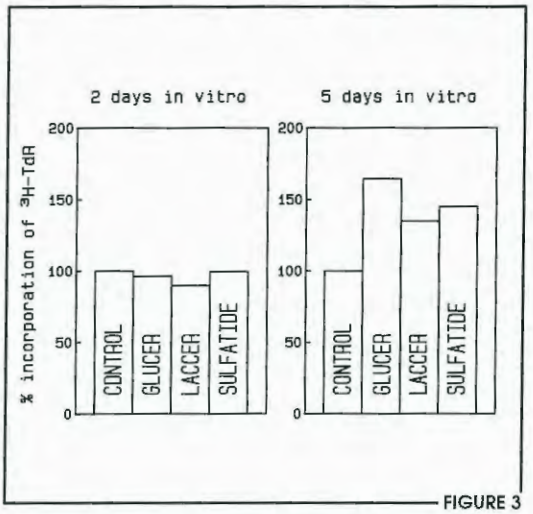

FIGURE 3

³H-thymidine ([³H] TdR) incorporation after treatment of human keratinocytes in culture with different glycolipids. [³H] TdR incorporation in keratinocytes not treated with glycolipids taken as 100% (control).
GLUCER = glucosylceramide; LACCER = lactosyl-ceromide.

Acknowledgments

This work was supported by a grant of C. N. R. (Rome, Italy; Progetto strategico: principi attivi per cosmetica).

References

1. **Gray G. M. and Yardley H. J. (1975)**: *J. Lip. Res.* **16**,434-440
2. **Gray G. M. and Withe R. J. (1978)**: *J. Invest. Dermatol.* **30**,336-341
3. **Schwartzmann G., Hoffmann-Bleihauer P., Shubert J., Sandhoff K. and Marsh D. (1983)**: *Biochemistry* **22**,5041-5048
4. **Chigorno V., Pitto M., Cardace G., Acquotti D. and Kirschner G. (1985)**: *Glycoconiugate J.* **2**,279-291
5. **K. L. Blacker, M. L. Williams and M. Goldyne (1987)**: *J. Invest. Dermatol.* **6**,536-539
6. **J. Hunyadi, M. Simon Jr. and A. Dobozy (1989)**: *Acta Derm. Venereol. (Stockh.)* **69**,509-512
7. **J. G. Rheinwald, H. Green (1975)**: Cell 6, 331-334