BIOLOGICALLY ACTIVE SUBSTANCES OF SKIN AFFECTING PROLIFERATION AND DIFFERENTIATION OF HUMAN KERATINOCYTES IN CULTURE

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The development of new therapeutic agents for use in the treatment of psoriasis is associated with the isolation and purification of dermal peptides, and also the creation of their synthetic analogs [11]. The leading mechanism of disturbance of proliferation of the epidermis in psoriasis is removal of the basal cells from the influence of the system of proliferative regulation. We know that many interdependent factors, including several synthesized by cells of the proliferating tissues themselves, are involved in the regulation of cellular proliferation [5, 13, 17]. With the development of epidermal cell culture techniques, test systems based on cultures of normal and malignant human and animal keratinocytes have achieved widespread popularity [8, 14, 18]. Numerous investigators have shown that epidermal cells in culture react to various regulators of cellular proliferation and differentiation, in the same way as epidermal cells in vivo [2, 6, 7]. Processes of growth predominate over differentiation in cultures of keratinocytes, which distinguishes them from the normal epidermis and more closely resembles the skin lesions of psoriasis [3]. The transition from proliferating cells in culture into the differentiated state is accompanied by a whole range of morphological and biochemical changes, characteristic of differentiation of the epidermis, namely the progressive increase in size of the cells, changes in the keratin profile, and the formation of ketatin membranes, due to activation of Ca^{2+}-dependent transglutaminase [4].

The aim of this investigation was to study the effect of biologically active substances of skin on growth and differentiation of human epidermal cells in culture.

EXPERIMENTAL METHOD

Keratinocytes were isolated and cultured by a modified method of Rheinwald and Green [15]. Human keratinocytes were isolated from a skin flap obtained after plastic operations, by incubation in 0.25% trypsin overnight at 4°C. After repeated trypsinization for 10-15 min the cell suspension was filtered through Kapron gauze and sedimented by centrifugation at 600 rpm for 3 min. The cells were resuspended in a mixture of media DMEM:F12 (1:1) with 10% fetal calf serum (FCS). Into each well of a 24-well planchet, containing human fibroblasts, previously seeded as feeder cells (10,000 cells per well) was added 1 ml of a suspension of keratinocytes (50,000 cells). On the 2nd day after seeding the original medium was replaced by a balanced medium containing epidermal growth factor (10 ng/ml), insulin (5 μg/ml), and isoproterenol (10^{-6} M). The level of cellular proliferation was assessed by determining the number of cells growing after 14 days. The cells with the test preparations were cultured by adding fractions (F_1, F_2, F_3) on the 2nd day after seeding in medium DMEM/F12 with 10% FCS, and with subsequent changes of medium. Control cultures were grown on balanced medium. The cells were removed by incubating the cultures in a mixture of 0.4% trypsin/0.02% EDTA for 30 min at 37°C. The experimental results were obtained by

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TABLE 1. Effect of Biologically Active Skin Fractions on Proliferation of Keratinocytes in Culture

Fraction, μg/ml	Number of cells	
	thousands/well	%
Control	562±54	100
F_1, 100	661±61	118
10	603±56	107
1	566±49	101
F_2, 100	438±40	78
10	548±59	97
1	560±48	100
F_3, 100	629±69	112
10	585±51	104
1	569±62	101

TABLE 2. Effect of Biologically Active Substances of Skin on Growth and Differentiation of Keratinocytes (in a test system including a stage of stripping)

Fraction	Number of cells with membrane		Number of basal cells	
	thousands/well	%	thousands/well	%
Control	34±3	6,0	91±9	16,2
F_1	55±6	8,3	88±7	13,3
F_2	67±8	15,3	40±5	9,1
F_3	30±4	4,8	91±8	14,5

counting the number of cells in 3-6 wells in a hemocytometer. Differentiation of the keratinocytes were evaluated by their ability to form keratin membranes [16]. For this purpose a suspension of cells removed from each well was sedimented by centrifugation and the residue of cells was treated with 1% SDS, 20 mM DTT for 20 min at room temperature. After centrifugation the number of corneas was counted in the hemocytometer, using a phase contrast microscope. In the 2nd version of test system cultures of epidermal cells were grown by introducing the preparations (100 μg/ml) into medium DMEM/F12 with 10% FCS on the 2nd day after transplantation of the cells, and at later changes of medium. Control cultures were grown on balanced medium.

After 18 days of culture stripping was carried out, by incubating the stratified cultures on medium DMEM without calcium ions and without sera for 3 days. The total number of growing cells was determined in the cultures before stripping. After stripping the number of cells possessing a keratin membrane and the number of basal cells, i.e., cells left on the growth surface after removal of differentiated layers of cells, were determined in the cultures. To determine the percentage of cells with a keratin membrane the number of detergent-resistant membranes was expressed as a ratio of the total number of cells in epidermal cultures before stripping. During simulation of regeneration of the epidermis cultures of keratinocytes were grown on balanced medium for 18 days and then stripped. After separation of the differentiated layers of cells with an aliquot of Hanks's solution without calcium ions, the basal cells were cultured in medium DMEM/F12 with the test fractions (100 μg/ml). Some cultures were kept on balanced medium, the control cultures on medium DMEM1/F12. After 1 day [^{3}H]-thymidine was added and its incorporation into DNA determined by adding [^{3}H]-methylthymidine in a concentration 1 μCi/ml to the culture medium and incubating for 18 h. After precipitation with TCA the level of radioactivity of the samples was measured on a Tracor Analytic counter (USA). To determine viability the suspension of keratinocytes was incubated with the F_2 preparation in different concentrations (50-200 μg/ml) for 2 h. Viability of the cells was estimated as ingestion of a vital dye, using a 0.5% solution of trypan blue. Biologically active substances from animals' skin were isolated by the method in [1]. As a result of purification of the skin extract three fractions were obtained: F_1 with mol. wt. of over 15 kD, F_2, with mol. wt. of 1.4 to 15 kD, and F_3 with mol. wt. of under 1.4 kD.

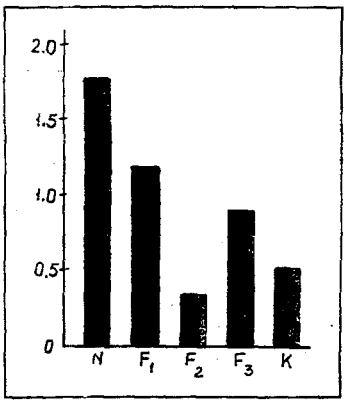

Fig. 1. Effect of biologically active substances of skin on proliferation of keratinocytes in culture in a model of regeneration of epidermis in vitro (incorporation of [^{3}H]-thymidine by keratinocytes during incubation: N) in normal medium; F_1, F_2, F_3) in medium DMEM/F12 containing test preparations, K) in medium DMEM/F12).

EXPERIMENTAL RESULTS

The effect of the biologically active fractions on proliferation and differentiation of the human keratinocytes in culture was studied by determining the total number of growing cells as a result of culture in medium containing the preparations, relative to the number of basal cells and the number of cells possessing a keratin membrane in the cultures subjected to stripping, and also as incorporation of [^{3}H]-thymidine into the DNA of the cells in a model system of regeneration of the epidermis.

In the 1st version of a test system the cells were cultured in the presence of the test preparations, and the number of growing cells and the number of cells with a detergent-resistant membrane were determined after 14 days. Preparations of all three fractions, in a concentration of 1-10 μg/ml of growth medium had no significant effect on growth of the keratinocytes (Table 1), but in a concentration of 100 μg/ml the preparation caused a decrease in proliferation of the keratinocytes. Preparations F_1 and F_3 in a concentration of 100 μg/ml had a stimulating action on proliferation of the epidermal cultures. In this system of culture the percentage of cells possessing a keratin membrane was only 1-2%. In view of the scatter of the data from one experiment to another, this value was not a reliable estimate of the effect of the preparations on differentiation of keratinocytes.

Viability of the epidermal cells as a result of incubation with the F_2 preparation in different concentrations revealed no disturbances of metabolism or of the structural integrity of the membranes. Consequently, it can be postulated that the effect of the preparation on the keratinocytes in culture is not cytotoxic.

To discover the effect of the preparations on differentiation of epidermal cells the test system was modified by introduction of a stripping stage, for the compulsors stripping of the mature epidermal cells.

A change in the extracellular calcium ion concentration has a marked effect on proliferation and differentiation of epidermal cells [9]. Culture of keratinocytes in medium with a low calcium ion concentration (0.05-1.0 mM) maintains them in an undifferentiated proliferating state. Conversely, if the culture is grown in medium with a normal calcium concentration (1.2 mM) all the signs of differentiation of the keratinocytes are found: stratification, an increase in the number of cells with a keratin membrane and containing keratohyaline granules, tonofilaments, and fibrous inclusions [12].

The "stripping" method is as follows: dense multicellular layers of an epidermal culture, grown on balanced medium, are transferred for 3 days to medium not containing calcium ions [10]. As a result an apparent maturation of the cells which have begun differentiation takes place, with detachment of the mature layers of cells. Under these circumstances a delicate monolayer of basal cells remains on the surface of the culture vessel. The content of cells

possessing a keratin membrane increases after stripping, and in this test system it is possible to estimate reliably the effect of the preparations on differentiation of the keratinocytes.

The results of this series of experiments show (Table 2) that the F_2 preparation stimulates differentiation of keratinocytes in culture. Determination of the content of basal cells in this test system revealed a decrease in the fraction of these cells in the total number of cells growing in the presence of the F_2 preparation. This fact is evidence of a decrease in proliferative activity of the epidermal culture under the influence of the F_2 preparation.

The 3rd version of a test system for testing modulators of growth and differentiation of epidermal cells, used in the work, is a model of regeneration of the epidermis in vitro [10]. It was shown that cultures of human keratinocytes, after detachment of the upper differentiating cell layers as a result of stripping in a balanced growth medium regain their proliferative activity and develop as a normal culture, followed by commencing differentiation. This reaction of the epidermal culture is similar to regeneration of the epidermis in vivo.

In these experiments the test factors were incubated with cultures of keratinocytes in the composition of media containing neither serum nor growth factors, so that the pure effect of these preparations alone on growth of epidermal cells can be evaluated. It will be clear from Fig. 1 that the F_1 and F_3 preparations behaved as growth stimulators, although their effect was less marked than that of the balanced medium containing serum and growth factors. As in the previous series of experiments, in the model of regeneration of the epidermis the F_2 preparation had an inhibitory effect on proliferation of the keratinocytes.

The results of the experiments conducted in three test systems lead to the conclusion that the F_2 preparation contains regulators of keratinocyte metabolism which inhibit proliferation and stimulate differentiation of the epidermal cells, whereas the F_1 and F_3 preparations potentiate the proliferative activity of these cells. It is intended in the future to continue these investigations with a view to isolating active peptides from these preparations and determining their effect in test system developed on the basis of keratinocytes in culture.

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