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SHORT REPORTS

Cryopreserved 3T3 Fibroblasts Retain Their Capacity to Enhance the Growth of Human Keratinocyte Cultures

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Cultured epithelial grafting of full-thickness skin defects is a new promising possibility for the successful treatment of patients with large burns. The major obstacle to this method, however, is a 3–4-week interval between burn and grafting, which is necessary for the growth of sufficient quantities of cultured epithelium. Generally, growth-arrested murine 3T3 fibroblasts have been used with success as feeder layers to shorten the cultivation time of keratinocytes. In the present work we have initiated studies to determine whether or not cryopreserved growth-arrested 3T3 fibroblasts retain their capacity to enhance the growth of human keratinocytes in vitro. The results of this study show that the [^{3}H]thymidine incorporation in cultures containing mitomycin C treated 3T3 feeder cells was significantly greater than in cultures without feeder cells. Furthermore, in keratinocyte cultures containing freshly separated or cryopreserved 3T3 fibroblasts, a similar rate of [^{3}H]thymidine incorporation was observed and the activity of incorporation has never differed significantly during the 11 days of culturing, meaning that cryopreserved, growth-arrested 3T3 fibroblasts retain their ability to enhance the growth of human keratinocytes. This observation renders the continuous maintenance of 3T3 cells unnecessary in laboratories which want to culture keratinocytes without delay. ***Key words: Epidermal cells; Burn; Skin.***

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The healing process of full-thickness skin defects can be accelerated effectively by autotransplantation. Since donor sites for autografting are often not available, the temporary coverage of wounds with material other than the patient's own skin has become common in recent decades (1). Fresh or cadaver skin allografts from related or unrelated donors are the most frequently used, and the most effective biological dressing. However, since skin allografts are rejected in about 2 weeks, regrafting is necessary (2). As a recent approach, cultured autologous skin epithelium has been used for grafting (3). The major obstacle for the use of cultured autografts, however, is a 3–4-week interval between burn and grafting, which is necessary for growth of sufficient quantities of epithelium.

In contrast to human dermal fibroblasts, murine fibroblasts (3T3) are reported to enhance the culturing of human keratinocytes (4). Accordingly, in the laboratories where keratinocyte cultures for transplantation are prepared, the importance of urgent cultivation means that an undetermined quantity of 3T3 cells must always be promptly available. In the present work we have initiated studies to determine whether or not cryopreserved, growth-arrested 3T3 fibroblasts retain their capacity to enhance the growth of human keratinocytes in vitro. The results of these studies show that cryopreservation fails to alter the stimulating ability of 3T3 cells as compared with that of freshly cultured 3T3 cells under similar conditions.

MATERIAL AND METHODS

Preparation of epidermal cell suspension

An epidermal cell (EC) suspension was prepared by a trypsin digestion method from full-thickness fresh skin specimens from healthy individuals who underwent plastic surgery (5). Briefly, after removal of the subcutaneous tissue and as much dermis as possible, the tissue was minced and trypsinized (Gibco, North Andover, Massachusetts, 0.25%, 12 h, 4°C) to produce a single-cell suspension. The cells were washed three times in phosphate-buffered saline (PBS; 0.05 M phosphate buffer, pH 7.2, and 0.1 M sodium chloride). The EC suspension contained 70–80% viable keratinocytes.

3T3 fibroblasts

Murine 3T3 cells (Flow Laboratories GmbH, Meckenheim, Kat.-Nr.: 03-405-84) were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco) containing 10% foetal calf serum (FCS) (Gibco). Confluent cultures of 3T3 cells were treated with mitomycin C (8 µg/ml) in PBS for 6 h, washed

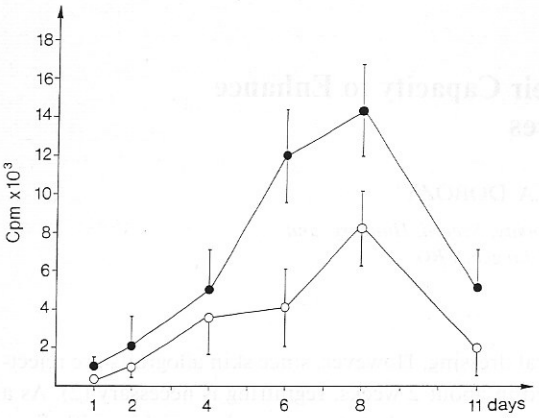

Fig. 1. Thymidine incorporation-enhancing effect of 3T3 fibroblasts in human epidermal cell (*EC*) cultures. ECs were cultured without (○) or with (●) freshly separated mitomycin C-treated 3T3 fibroblasts for 11 days. The activity of [^{3}H]TdR incorporation was determined as indicated at different times after initiation of the cultures. Each point represents the mean ± SE derived from triplicate determinations of four experiments.

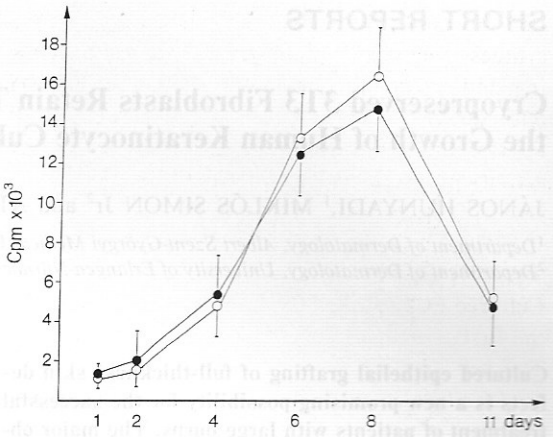

Fig. 2. Ability of cryopreserved 3T3 fibroblasts to enhance the growth of cultured human keratinocytes. Epidermal cells (*EC*s) were cultured together with freshly separated (●) or cryopreservd (○) mitomycin C-treated 3T3 fibroblasts for 11 days. The activity of [^{3}H]TdR incorporation was determined as indicated at different times after initiation of the cultures. Each point represents the mean ± SE derived from triplicate determinations of four experiments.

twice with PBS, and harvested with 0.1% trypsin in PBS to detach the cells from the culture dish.

Freezing of 3T3 fibroblasts

Mitomycin-C treated 3T3 cells were frozen in DMEM containing 7.5% FCS and 25% dimethylsulphoxide.

Cell cultures

Human ECs were cultured as described by Rheinwald & Green (6). For tissue culturing, the separated cells were inoculated at a density of 10^4 cells/cm^2 into 25 cm^2 Falcon rectangular flasks or into 96-well flat-bottomed microtitre plates (Nunc, Roskilde, Denmark). Freshly separated or cryopreserved mitomycin C-treated 3T3 cells were added to the cultures with a density of 10^5 cells/cm^2 4 h prior to initiation of the EC cultures. The growth medium (10 ml or 0.2 ml) for all cell cultures consisted of a 3:1 mixture of DMEM and Ham's F-12 medium (Boehringer, Mannheim GmbH, West Germany) supplemented with 10% FCS, 0.4 µg/ml hydrocortisone (Sigma, St Louis, Missouri), 5 µg/ml insulin (Sigma), 5 µg/ml transferrin (Sigma), 2×10^{-9} M triiodothyronine (Sigma), 1 nM cholera toxin (Sigma), and 1.8×10^{-4} M adenine (Sigma). 48 h after initiation and subsequently every second day, the cultures were fed with the same medium containing 10 ng/ml epidermal growth factor (EGF) (Sigma). For the harvesting of differently aged cultures (1, 2, 4, 6, 8 and 11 days) six parallel plates were prepared in all experiments.

Thymidine incorporation assay

The stimulating efficacy of the 3T3 cells was assessed by monitoring the [^{3}H]thymidine ([^{3}H]TdR) incorporation of the EC cultures at different times after initiation. Cultures were pulsed with 2 µCi/well [^{3}H]TdR (Amersham International PLC) and cultured for an additional 6 h, after which period the cells were harvested for beta scintillation counting.

RESULTS

Efficacy of 3T3 fibroblasts to stimulate human keratinocytes

The kinetics of DNA synthesis in cultures without 3T3 fibroblasts was similar to that in cultures containing freshly separated mitomycin-C treated 3T3 feeder cells. In contrast, the degree of [^{3}H]TdR incorporation was markedly different. During the first 4 days, there was no statistically significant difference in [^{3}H]TdR incorporation between the two kinds of EC culture. Later, the rate of DNA synthesis increased rapidly under both conditions, to reach its peak on the 8th day after initiation. Subsequently, a marked decrease in [^{3}H]TdR incorporation was observed. After the 4th day of cultivation, the DNA synthesis in cultures containing feeder cells was significantly higher than that in EC cultures without 3T3 fibroblasts (Fig. 1).

Efficacy of cryopreserved 3T3 fibroblasts to stimulate human keratinocytes

In EC cultures containing freshly separated or cryopreserved feeder cells, a similar [^{3}H]TdR incorpora-

tion was observed. Under both conditions, the DNA synthesis increased rapidly after the 4th day of culturing, and reached the maximum level on the 8th day. Subsequently, a decrease in activity was measured. In the two kinds of culture, the [^{3}H]TdR incorporating activity never differed significantly (Fig. 2).

Behaviour of epidermal cell cultures during cultivation

Cultured ECs attached themselves to the dish within the first 2 days of cultivation and formed colonies. The cell colonies fused to each other and spread gradually. ECs cultured without 3T3 cells did not become confluent during the 11 days of cultivation. On the other hand, keratinocytes cultured together with freshly separated or cryopreserved 3T3 cells became confluent on the 8th day of culturing.

In some representative experiments, ECs were cultured in the presence of cryopreserved 3T3 feeder cells in 25 cm^2 Falcon flasks for morphological investigations. The cultured epithelia were detached from the dish completely, by using 1.2 U/ml dispase II. Histological and electronmicroscopic examination of the sheets showed that the cultured epithelia were composed of 2–4 layers of ECs.

DISCUSSION

The grafting of autologous separated ECs or cultured epithelium onto various smaller skin defects, such as ulcers, burns and surgical skin defects, has proved satisfactory (7–9). The most spectacular results were obtained in patients with subtotal body surface burns and in children with giant congenital nevi (10–13).

In the isolation process of keratinocytes for culturing, the epidermis is separated from the deeper layers of the skin by dissection or trypsinization, and the epidermis is then disaggregated in a further trypsinization (5). Methods for growing the ECs, prepared in this way fall into two broad categories: those which allow serial passage from low inocula (6), and those which attempt primary culturing (14). In the first category, human keratinocytes are seeded onto a feeder layer of mouse 3T3 fibroblasts, which conditions the culture medium, encouraging attachment and fast proliferation (6). Accordingly, in the present work we observed that EC cultures formed a confluent sheet much faster and showed a significantly increased [^{3}H]TdR incorporation in the presence of the 3T3 feeder cells (Fig. 1).

In urgent situations, such as subtotal body burns, the duration of cultivation is the rate-limiting factor of successful autotransplantation (15). The present study has provided evidence that cryopreserved, growth-arrested 3T3 fibroblasts retain their ability to enhance the growth of human keratinocytes (Fig. 2). This observation renders the continuous maintenance of 3T3 cells unnecessary; if cryopreserved cells are used, the EC cultures can be initiated without delay.

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Two Binding Sites for Ki67 Related to Quiescent and Cycling Cells in Human Epidermis

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The monoclonal antibody Ki67 (Ki67) binds to a nuclear antigen expressed by cycling cells of several human tissues and to the cytoplasm of the basal layer cells of squamous epithelia. We have used an immunohistochemical method to visualize the binding sites of Ki67 in normal and hyperproliferative epidermis. Cytoplasmic staining was present in the basal layer cells of normal epidermis, but was decreased in psoriatic and post-tapestripping epidermis. In sections of normal epidermis only a small minority of nuclei were positive, but sections of psoriatic epidermis and epidermis 40 and 48 h after tapestripping showed large numbers of positive nuclei in the basal and suprabasal layers. Since recent reports strongly suggest that the cell production rate is regulated by changes in the number of cycling cells, the hypothesis that Ki67 binds also in human epidermis to the nuclei of cycling cells is supported by the present findings. *Key words: Monoclonal antibody Ki67.*

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The monoclonal antibody Ki67 was originally raised against a nuclear substance of Hodgkin lymphoma cells, which is also expressed by actively cycling cells of several other human tissues (1, 2). The hypothesis that the nuclear binding of Ki67 in epidermis is associated with actively cycling cells is based on analogy with these tissues. At least in human leukocytes, the nuclei of S, G_2, M and G_1 cells were positive for Ki67, but those of G_0 cells negative. Cells entering the cell cycle from a G_0 state became positive before entering the S-phase but after increased RNA amounts, typical for G_1, were detected; this indicates that after quiescence the S-phase is not preceded by a common G_1 phase (3). The discrimination between actively cycling and quiescent (G_0) cells is of very great general interest because it allows the quantification of growth fractions. It is especially important for human epidermis because the existence of quiescent (G_0) cells is still not generally accepted and the relative number of cycling cells has never been directly determined.

However, this is only one aspect of Ki67. According to the product information supplied by the manufacturer (Dakopatts), Ki67 also binds to a cytoplasmic structure in the basal layer of squamous epithelia such as epidermis. This binding site also appears to be related to proliferation. We have developed an immunohistochemical procedure for human epidermis and applied it to biopsies of normal, psoriatic and tapestripped skin, in order to determine the proportion of Ki67-positive cells and to investigate the expression of the cytoplasmic binding site under these circumstances.

MATERIALS AND METHODS

Sample procedure

Shave biopsies were taken using a standard technique from normal skin of healthy volunteers, from the borders of previously tapestripped areas (16, 32, 40 and 48 h after stripping) on the backs of healthy volunteers and from the border of an untreated lesion of a psoriatic patient. Within 15 min,