Acta Derm Venereol (Stockh) 1991; 71: 195–198

Inhibition of Basal Cell Proliferation during Storage of Detached Cultured Epidermal Keratinocyte Sheets

Y. POUMAY, F. BOUCHER, A. DEGEN and R. LELOUP

Department of Histology-Embryology, Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium

Grafting of human cultured keratinocytes is a promising possibility for the treatment of large burned skin areas. However, during the preparation of cultures for grafting, a necessary step of detachment from culture substratum is performed by incubation with Dispase. In the present paper, we report studies performed to determine whether prolonged storage after detachment would have a detrimental effect on keratinocyte proliferation. Our results show that the number of bromodeoxyuridine-incorporating cells located in the basal layer of the culture gradually diminishes during storage of detached cultured epidermal keratinocyte sheets. Furthermore, the rate of [^{3}H]thymidine incorporation in such detached cultures is also progressively reduced. These observations indicate that the detachment and storage of cultured epidermal keratinocyte sheets for grafting impedes their cell proliferation capacity, as revealed by DNA-synthesis measurements. ***Key words: Cell adhesion; Burn; Dispase.***

(Accepted October 1, 1990.)

Acta Derm Venereol (Stockh) 1991; 71: 195–198.

Y. Poumay, Département d'Histologie-Embryologie, Facultés Universitaires Notre-Dame de la Paix, Rue de Bruxelles, 61, B-5000 Namur, Belgium.

Cultured epidermal keratinocyte (EK) sheets are considered by many burn centres to be suitable material for the temporary coverage of wounds when used as keratinocyte allografts (1) or for permanent coverage when used as autografts (2–4). Other uses exist and involve supplementation of spliced full skin autograft with cultures of keratinocytes, the assumption being that beneficial diffusible products are secreted by these cultured epidermal cells (5). Thanks to the various advantages afforded by EK cultures, an increasing number of burn centres are employing these relatively fragile sheets (6) which means that clinical management needs to be studied, for example in the use of topical antimicrobial agents (7).

Before the burned area is covered, the EK culture is processed through several steps. Amongst these, the necessary step of epidermal sheet detachment from culture substratum by Dispase, as proposed by Green et al. (8), has no detrimental effect on the keratinocytes regarding cell proliferation. Indeed, this enzyme does not damage cell membranes or intercellular junctions, as Dispase is a powerful fibronectinase and type IV collagenase (9). However, when a detached epidermal sheet has to wait before wound application, basal keratinocytes that have grown in close contact with substratum and basement membrane components are then located without any anchorage on one surface of the EK sheet.

The aim of our study was to assess whether this location of basal cells could have an injurious effect, when prolonged for some hours, on their proliferation potential.

MATERIAL AND METHODS

Culture of keratinocytes

Epidermal keratinocyte suspensions were prepared by a trypsinization method from healthy human skin specimens removed during surgery. Briefly, the tissues were incubated in a trypsin (0.25%, Difco, Detroit, Mich., USA) solution for 60 min at 37°C. After detachment of the epidermis from the dermis, a keratinocyte suspension was prepared by numerous passages in a pipette. After centrifugation, the EK were grown in the presence of a feeder layer of mitomycin C-treated 3T3 cells (8). The culture medium contained 1 part Ham's F12 medium plus 3 parts Dulbecco's modified Eagle medium. This was supplemented with 10% fetal calf serum (Gibco, Ghent), 5 µg/ml insulin (Sigma, St. Louis, Mo., USA), 0.4 µg/ml hydrocortisone (Sigma), 10^{-10} M cholera toxin (Sigma), 5 µg/ml transferrin (Sigma), 2×10^{-9} M triiodothyronin (Sigma), 1.8×10^{-4} adenine (Sigma) and 10 ng/ml epidermal growth factor (EGF) (Gibco). At confluence, the EK were trypsinized with a solution of trypsin (0.1%) : EDTA (0.02%). They were then plated on plastic 6 multiwell plates (2×10^5 cells per well) in the presence of the same feeder layer in order to obtain 7–8 days later a secondary confluent EK culture.

Detachment of EK cultures

Confluent EK cultures were treated with Dispase II (Boehringer Mannheim) at 1.2 U/ml in a serum-free medium for 1 h at 37°C (8). After detachment, the sheets were rinsed twice in phosphate-buffered saline (PBS) and then replaced in culture medium at 37°C and stored for 0 to 24 h before testing the DNA synthesis.

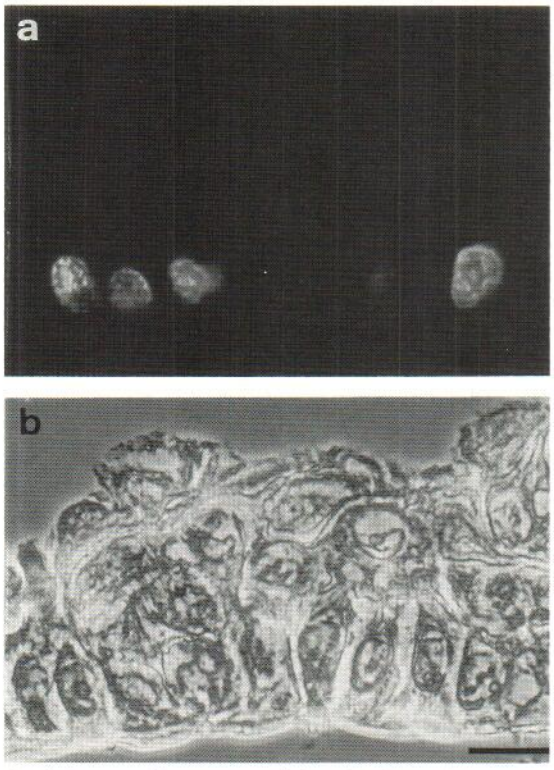

Fig. 1. Immunofluorescent staining of BrdU-positive nuclei (*a*) and the same field observed by phase-contrast microscopy (*b*) of a perpendicular section from a detached EK sheet (bar, 20 μm).

5-Bromo-2'-deoxyuridine (BrdU) incorporation and immunolabelling

After storage in their culture medium for 0, 2, 4, 6, 8, 12 or 24 h, detached EK sheets were incubated in the same medium containing 10 μM BrdU (Sigma) for 1 h at 37°C. These sheets were then washed in PBS before fixation in 70% ethanol (1 h, 4°C) and embedding in paraffinwax. Perpendicular sections (6 μm) were immunolabelled for the detection of BrdU incorporation: after rehydration, cellular DNA was denatured with 2 N HCl (30 min, 37°C) and neutralized with 2 washes in 0.1 M sodium tetraborate solution (pH 8.5); slides were then covered for 1 h at 37°C in a humidified chamber with 75 μl of anti-BrdU monoclonal antibody (Becton Dickinson, Mountain View, Calif., USA) at a 1 : 10 dilution in PBS. After three washes in PBS, slides were incubated in the same conditions with FITC-conjugated F(ab')2 fragment of rabbit IgG to mouse IgG (Dakopatts, Denmark) at a 1 : 20 dilution in PBS. After three washes in PBS, the sections were mounted in Glycergel (Dakopatts) and fluorescent nuclei containing cells were examined by epifluorescence microscopy under a Zeiss Photomicroscope equipped with RS-III epicondensor and alternatively localized using phase contrast microscopy. For the counting of the total number of nuclei and labelling index determination (percentage of labelled nuclei), sections were stained lightly with propidium iodide (Sigma). A sufficient number of fields were screened in order to be able to count at least 500 nuclei per section.

Thymidine incorporation assay

After 0, 2, 4, 6 or 8 h of detachment, the [^{3}H]thymidine incorporation of EK cultures was monitored by a 60 min incubation at 37°C with 2 μCi/graft [*methyl*-^{3}H]thymidine (Amersham, Belgium), followed by beta scintillation counting of the radioactivity incorporated into trichloroacetic-precipitable cellular material (10).

Cell lysis measurement

EK lysis was checked by measurements of released lactate dehydrogenase (LDH) activity as described elsewhere (11).

RESULTS

Localization of proliferating keratinocytes in a confluent culture

The localization of proliferating cells was made possible by the immunofluorescent labelling of BrdU-positive nuclei in perpendicular sections, together with phase-contrast microscopy in recently detached EK sheets (Fig. 1). The previously anchored basal layer can be identified by its characteristic polygonal cell shape obtained after detachment. We demonstrate here that cells that are synthesizing DNA in an EK culture are only present in the basal layer. This is identical in cultures incubated with BrdU before detachment and has been compared with the localization in *in vivo* human epidermis where proliferating cells are predominantly shown in the first suprabasal layer (not shown).

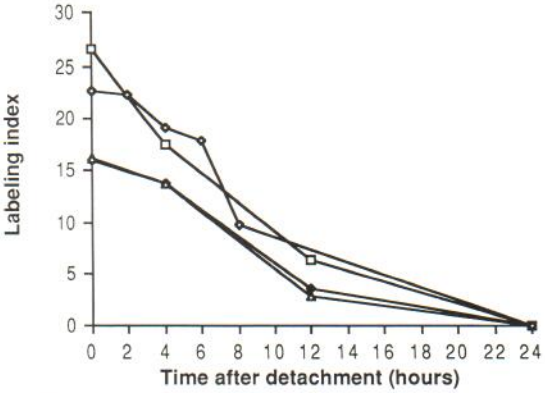

Fig. 2. Labelling index determined by BrdU incorporation in EK cultures obtained from four different skin specimens and after increasing storage periods following detachment.

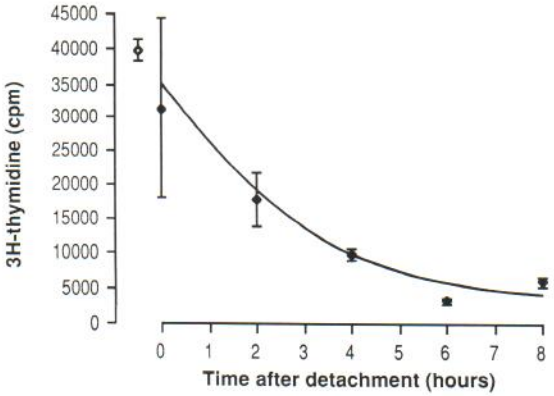

Fig. 3. [^{3}H]thymidine incorporation revealed after increasing storage periods following detachment (●) or before detachment (○) of the cultured EK sheets. Results are expressed as means ± SE of triplicates.

Effect of storage duration on the labelling index (BrdU incorporation) of detached EK sheets

The labelling index was determined in detached EK sheets obtained by the culture of keratinocytes derived from four different skin specimens and after progressively increased storage periods. These results are illustrated in Fig. 2. This labelling index decreases progressively within 12 h after detachment and no labelling can be detected after 24 h. Furthermore, the fluorescent nuclei observed after 8 and 12 h of storage appear to be very faintly labelled, although all procedural conditions remain constant.

Radioactive thymidine incorporation after 0–8 h of storage of detached EK sheets

The detachment of the confluent culture does not by itself produce a significant modification in the degree of [^{3}H]thymidine incorporation, although it seems to induce an increased variability. On the other hand, an increased time after detachment (2, 4, 6 or 8 h) progressively inhibits [^{3}H]thymidine incorporation (Fig. 3). According to these results, the DNA synthesis could rapidly be reduced to less than 50% of that observed in non-detached EK cultures. This inhibition is more rapidly obtained than the decrease in the labelling index. This result has to be related to our observation that the incorporation of BrdU seems to be considerably reduced, though still detectable, in basal keratinocytes of sheets stored for 8 h or more after detachment. Indeed, since the labelling index reflects the proportion of BrdU-positive cells, it does not depend on the rate of nucleotide incorporation, but depends on whether the incorporation still occurs or not.

In similar conditions, the released LDH activity was determined after 0, 4 and 8 h of incubation and revealed 1.1%, 6.3% and 11.4% respectively of mean cell lysis.

DISCUSSION

The grafting of cultured human EK in various situations has afforded very satisfactory results (2–6) and has therefore been adopted by many burns centres. However, utilization of EK means that confluent cultures have to be harvested as thin epidermal sheets by detachment from culture substratum with Dispase (8, 9). Since the preparation for grafting of several sheets from confluent cultures cannot be concomitant, it is often necessary to store the first detached EK sheets during the processing of subsequent cultures. the present work was therefore performed to assess whether basal keratinocytes originally anchored during the culture could still proliferate after detachment with Dispase.

In culture, we show first that DNA-synthesizing cells are located in the basal layer, in contrast with the epidermis where a majority are found in the first suprabasal layer (12, 13). When storage takes place after detachment of the culture, the rapid decrease in radioactive thymidine incorporation, coupled with the decreased number of BrdU labelled cells, suggests that, in our experiments using confluent EK cultures, anchorage to substratum is necessary in order to maintain the basal EK proliferation. This dependence of human EK proliferation on cell-substratum adhesion has already been reported for isolated cells cultured in suspension or on restricted area of substratum contact (14). It was then suggested that cell shape controls important EK functions: the proliferation of a rounded cell is inhibited, whereas it is preserved in a spread cell. It is therefore interesting to note that, despite the fact that our results were obtained with detached confluent EK cultures, the inhibition of basal cell proliferation could also be the result of a change in the cell's shape. Indeed, Dispase detachment of EK cultures induces fully spread basal cells to become more spherical (15). Lastly, at first sight, these decreased measurements cannot be explained solely by cell lysis, since lysis is less than 12% after 8 h of detachment. However, lysed cells could be those originally proliferating in the basal layer, since numerous bleb

formations are then borne by these cells as observed by light and electron microscopy (not shown).

These preliminary observations demonstrate that after detachment by Dispase, keratinocytes embedded in a multilayer culture are progressively disturbed by these conditions. Therefore, the storage of detached cultured EK sheets has to be kept as short as possible in order to preserve the basal cell's proliferation capacity. An alternative solution to these problems of EK sheets detachment would be the use of a wound covering made of epidermal and dermal components transplanted *en bloc* (16). However, this technique has not yet been demonstrated to be the most suitable for rapid covering of large wounds (6).

ACKNOWLEDGEMENTS

The authors wish to thank Mrs F. Zune-Mossoux, Mr R. Deom and Mr M. Lombet for technical assistance. Special thanks are addressed to Dr M. Paye, Dr E. Ketz, and Dr A. Vanderkelen of Brussels Military Hospital for many helpful discussions and for providing skin specimens. This work was supported by a grant from the Aide Sociale aux Grands Brûlés.

REFERENCES

1. Brain A, Purkis P, Coates P, Hackett M, Navsaria H, Leigh IM. Survival of cultured allogenic keratinocytes transplanted to deep dermal bed assessed with probe specific for Y chromosome. Br Med J 1989; 298: 917–919.
2. Gallico GG, O'Connor NE, Compton CC, Kehinde O, Green H. Permanent coverage of large burn wounds with autologous cultured human epithelium. N Engl J Med 1984; 311: 448–451.
3. Pittelkow MR, Scott Re. New techniques for the in vitro culture of human keratinocytes and perspectives on their use for grafting of patients with extensive burns. Mayo Clin Proc 1986; 61: 771–777.
4. Compton CC, Gill JM, Bradford DA, Regauer S, Gallico GG, O'Connor NE. Skin regenerated from cultured epithelial autografts on full-thickness burn wounds for 6 days to 5 years after grafting. Lab Invest 1989; 60: 600–612.
5. Hancock K, Leigh IM. Cultured keratinocytes and keratinocyte grafts. Skin grafts from the laboratory can supplement autografts. Br Med J 1989; 299: 1179–1180.
6. Woodley DT. Covering wounds with cultured keratinocytes. J Am Med Assoc 1989; 262: 2140–2141.
7. Cooper ML, Boyce ST, Hansbrough JF, Foreman TJ, Frank DH. Cytotoxicity to cultured human keratinocytes of topical antimicrobial agents. J Surg Res 1990; 48: 190–195.
8. Green H, Kehinde O, Thomas J. Growth of cultured human epidermal cells into multiple epithelia suitable for grafting. Proc Natl Acad Sci USA 1979; 76: 5665–5668.
9. Stenn KS, Link R, Moellmann G, Madri J, Kuklinska E. Dispase, a neutral protease from *Bacillus polymyxa*, is a powerful fibronectinase and type IV collagenase. J Invest Dermatol 1989; 93: 287–290.
10. Russel SB, Trupin KM, Rodriguez-Eaton S, Russel JD, Trupin JS. Reduced growth-factor requirement of keloid-derived fibroblasts may account for tumor growth. Proc Natl Acad Sci USA 1988; 85: 587–591.
11. Poumay Y, Ronveaux-Dupal MF. Incubation of endothelial cells in a superoxide-generating system: impaired low-density lipoprotein receptor-mediated endocytosis. J Cell Physiol 1988; 136: 289–296.
12. Lavker RM, Sun TT. Epidermal stem cells. J Invest Dermatol 1983; 81: 121s–127s.
13. Régnier M, Vaigot P, Darmon M, Pruniéras M. Onset of epidermal differentiation in rapidly proliferating basal keratinocytes. J Invest Dermatol 1986; 87: 472–476.
14. Watt FM, Jordan PW, O'Neill CH. Cell shape controls terminal differentiation of human epidermal keratinocytes. Proc Natl Acad Sci USA 1988; 85: 5576–5580.
15. Banks-Schlegel S, Green H. Involucrin synthesis and tissue assembly by keratinocytes in natural and cultured human epithelia. J Cell Biol 1981; 90: 732–737.
16. Hansbrough JF, Boyce ST, Cooper ML, Foreman TJ. Burn wound closure with cultured autologous keratinocytes and fibroblasts attached to a collagen-glycosaminoglycan substrate. J Am Med Assoc 1989; 262: 2125–2130.