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Culture of Keratinocytes for Transplantation Without the Need of Feeder Layer Cells

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Patients with large burn wounds have a limited amount of healthy donor skin. An alternative for the autologous skin graft is transplantation with autologous keratinocytes. Conventionally, the keratinocytes are cultured with mouse feeder layer cells in medium containing fetal calf serum (FCS) to obtain sufficient numbers of cells. These xenobiotic materials can be a potential risk for the patient. The aim of the present study was to investigate if keratinocytes could be expanded in culture without the need of a feeder layer and FCS. Keratinocytes were cultured on tissue culture plastic with or without collagen type IV coating in medium containing Ultroser G (serum substitute) and keratinocyte growth factor (KGF). An in vitro skin equivalent model was used to examine the capacity of these cells to form an epidermis. Keratinocytes in different passages (P2, P4, and P6) and freshly isolated cells were studied. Keratinocytes grown on collagen type IV were able to form an epidermis at higher passage numbers than cells grown in the absence of collagen type IV (P4 and P2, respectively). In both cases the reconstructed epidermis showed an increased expression of Ki-67, SKALP, involucrin, and keratin 17 compared to normal skin. Only 50,000 keratinocytes grown on collagen type IV in P4 were needed to form 1 cm^2 epidermis, whereas 150,000 of freshly isolated keratinocytes were necessary. Using this culture technique sufficient numbers of keratinocytes, isolated from 1 cm^2 skin, were obtained to cover 400 cm^2 of wound surface in 2 weeks. The results show that keratinocytes can be cultured without the need of a fibroblast feeder layer and FCS and that these cells are still able to create a fully differentiated epidermis. This culture technique can be a valuable tool for the treatment of burn wounds and further development of tissue engineered skin.

Key words: Epidermis; Culture; Burn wounds; Collagen type IV; Skin equivalents

INTRODUCTION

The standard treatment for extensive burn wounds is transplantation with a meshed split skin autograft. However, this treatment has disadvantages. Due to limited availability of healthy skin, insufficient donor sites may be present in patients with extensive burns. In addition, severe scarring and morbidity of the donor sites may occur and the burn wound often heals with an irregular mesh pattern.

An alternative for the use of autologous skin grafts is transplantation with engineered skin substitutes (1). In recent years, both dermal and epidermal substitutes have been developed. Clinical studies have shown that the use of dermal substitutes, like Integra or Alloderm, in combination with a very thin split thickness skin graft provides a permanent cover that is at least as satisfactory as an autologous meshed split thickness skin graft alone (19). In addition, treatment with these dermal substitutes is associated with decreased length of hospital stay (11,36). In this procedure the split skin autograft can be widely meshed and for this reason a reduced amount of donor skin is required. In addition, the very thin grafts reduce the risk of donor site morbidity.

The first epidermal substitute was achieved by culturing autologous keratinocytes to form a confluent epithelial sheet. The keratinocytes used in this substitute were cultured according to the method described by Rheinwald and Green (33). With this technique keratinocytes are cocultured with irradiated mouse 3T3 feeder layer cells, using medium containing fetal calf serum (FCS) and epidermal growth factor (EGF). The cultured epithe-

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lial autografts (CEA) can restore epidermal coverage and can be life saving for patients suffering from extensive burns (26). During the following years, contradicting literature concerning the clinical usefulness of the CEA in burn wound care has been published. The take of the sheets is variable and often poor and there is a high chance of blistering (5). In addition, the grafts are fragile and it takes 3–5 weeks to culture the sheets.

An alternative for the transplantation with keratinocyte sheets is transfer of preconfluent keratinocytes. These proliferating cells have a better take rate than confluent sheets (14). In addition, the use of preconfluent keratinocytes decreases culture time and costs. Animal and clinical studies show that transfer of these cells to the wound bed can be accomplished by a variety of carrier systems such as fibrin (28), polyurethane membranes (32), porous synthetic carriers (44), and aerosol sprays (24). Such keratinocyte delivery systems are easier to handle than the epidermal sheets. For the coverage of large burn wounds this technique can be used in combination with meshed skin autografts, which supplies the keratinocyte and fibroblast stem cells. Zhu et al. (47) showed that keratinocytes cultured on a plasma polymer surface (PSS) in combination with a meshed split skin autograft accelerated reepithelialization and showed improved healing with a less visible mesh pattern. They also reported that application of the cultured keratinocytes to the donor sites accelerated healing.

The standard culturing technique to obtain sufficient numbers of keratinocytes is coculture with irradiated mouse 3T3 feeder layer cells in Green's medium containing 10% FCS. However, the use of mouse feeder layer cells or FCS is a potential risk for the patient, because of possible disease transfer (e.g., animal viruses or prions) and the possible immunological response in the patient (3,23). For this reason the European authority prefers xenobiotic-free culturing conditions for clinical applications. The latest proposal for a regulation on advanced therapy medicinal products, which includes tissue engineered products, states that member states should decide themselves whether to allow the use of animal cells or medicinal products based on such cells (31). However, very detailed information has to be provided about many aspects of the involved animals before the European authority approves the utilization of these products (4).

Several approaches have been developed to avoid the use of xenobiotic materials. Work by the group of MacNeil showed that murine feeder layer cells can be replaced by human dermal fibroblasts (16,40). Keratinocytes attach and proliferate well on PPS in combination with the human feeder cells. However, it takes some weeks before sufficient numbers of the fibroblasts are cultured and the cells can be used as a feeder layer. Smola et al. showed that FCS can be replaced by autologous patient serum in keratinocytes cultures (39). However, this is only successful in the presence of murine feeder layer cells. In addition, it is undesirable to draw blood from patients with large burn wounds, because these patients already have a fluid shortage.

It has been shown that the keratinocytes that adhere most rapidly to collagen type IV have characteristics of stem cells (17,18). The rapidly adhering keratinocytes had a high colony forming efficiency (CFE) and showed low expression of differentiation markers (17). In addition, culture on collagen type IV increases the life span of murine keratinocytes (12,30). We hypothesized that collagen type IV could also improve the culture of human keratinocytes.

The aim of our study was to isolate and to culture sufficient numbers of keratinocytes that are still able to regenerate an epidermis in a short time frame without the need of mouse feeder layer cells and FCS.

MATERIALS AND METHODS

Tissue Handling and Cell Culture

A 0.3-mm split thickness skin graft was obtained using a dermatome (Aesculap AG & Co. KG, Tuttlingen, Germany) from tissues donated by healthy donors undergoing abdominal dermolipectomy. Epidermis and dermis were separated by incubation in 0.25% (w/v) dispase II (Boehringer, Mannheim, Germany) in saline at 37°C for 1 h.

Keratinocyte Culture. Keratinocytes were isolated from the epidermis by trypsin digestion (0.05% trypsin with 0.53 mM EDTA; Invitrogen, Breda, The Netherlands) at 37°C for 15 min. Isolated keratinocytes were cultured in DMEM/Ham's F12 (3:1) (Invitrogen), supplemented with 1% Ultroser G (Pall BioSepra, Cergy-Saint-Christophe, France), 0.4 μg/ml hydrocortisone, 0.25 μg/ml isoproterenol, 5 μg/ml insulin, 1 ng/ml recombinant human KGF (all Sigma-Aldrich, St. Louis, MO), and penicillin/streptomycin (100 IU/ml penicillin, 100 μg/ml streptomycin; Invitrogen). Cells were plated (80,000 cells/cm^2) onto tissue culture plastic alone or tissue culture plastic coated overnight with 1 μg/cm^2 collagen type IV from human placenta (Sigma-Aldrich) at room temperature. Eighty-five percent confluent cells were trypsinized using trypsin/EDTA and seeded in new flasks at a density of 13,000 cells/cm^2.

Fibroblast Culture. Dermal fibroblasts were isolated from the dermis as described by van den Bogaerdt et al. (43) and cultured in fibroblast culture medium [DMEM supplemented with 2% Ultroser G, 1 mM L-glutamine (Invitrogen) and penicillin/streptomycin]. Fibroblasts

were seeded at a density of 5,000 cells/cm^2. Passage 2 fibroblasts from one donor were used to construct all full skin equivalents.

Amplification of Keratinocytes

To assess the amplification of cultured keratinocytes, cells were counted with a haemocytometer (Bürker, Omnilabo, The Netherlands) at isolation and after passaging. The amplification per passage number was defined as the number of harvested keratinocytes divided by the number of seeded keratinocytes. The amplification was defined for six independent cell isolations.

Reconstruction of Epidermis

De-epidermized dermis (DED) was prepared by incubating human cadaver skin (Euro Skin Bank, Beverwijk, The Netherlands) in PBS for 3 weeks at 37°C under continuous agitation by shaking. Subsequently, the epidermis was scraped off and the remaining dermis was thoroughly washed in PBS.

Fibroblasts (0.2×10^6) were seeded inside a stainless steel ring (diameter 1 cm) at the reticular side of the DED. After 3 h, the ring was removed and fibroblast culture medium was added. After 3 days, keratinocytes were seeded inside a stainless steel ring at the papillary side of the DED. The ring was removed after 4 h and the skin equivalents were cultured in culture medium previously described by Gibbs et al. (10).

After 4 days, the cultures were placed on a small stainless steel grid and lifted to the air–liquid (A/L) interface. After 7 days of culture at the A/L interface, KGF and EGF were omitted from the culture medium and the cultures were grown for an additional 7 days at the A/L interface. Culture medium was refreshed two times per week.

Immunohistochemistry and Immunofluorescence

Cultures were harvested, fixed in 4% formaldehyde and processed for paraffin embedding. Sections (5 μm) were cut, deparaffinized, and rehydrated in preparation for morphological analysis by hematoxylin and eosin staining or immunohistochemical analysis by staining for keratin 17 (K17), skin-derived antileukoproteinase (SKALP), involucrin, Ki-67, p63, and β_1-integrin. Table 1 shows specification of the monoclonal antibodies and dilution of the antibodies used. After incubation with primary antibodies, sections were incubated with secondary biotinylated rabbit anti-mouse polyclonal antibody (DAKO, Glostrup, Denmark) for 1 h at room temperature. Subsequently, the sections were stained with streptavidin-biotin-peroxidase complex system (DAKO) according to the manufacturer's instructions. All sections were counterstained with hematoxylin.

For immunofluorescence, sections were incubated with streptavidin-alkaline phosphatase (DAKO) after incubation of secondary antibody. Staining was visualized using Liquid Permanent Red (DAKO) according to the manufacturer's instructions. Fluorescence was detected by fluorescence microscopy using a Texas Red filter set (excitation wavelength 510–560 nm and emission wavelength 590 nm). 4′,6-Diamidine-2-phenylindole (DAPI; Roche Diagnostics, Almere, The Netherlands) was used as a nuclear counterstain. Negative controls were performed in the absence of primary antibody.

Proliferation Index

The proliferation index (PI) is defined as the number of Ki-67-positive basal cells divided by the total number of basal cells × 100%. Staining of Ki-67 was equally distributed in the epidermis. Cells were counted at the light-microscopic level (magnification 100×) at three randomly chosen regions in each section. The resulting values are expressed as the median of three independent experiments.

Statistical Analysis

All data are expressed as the median of at least three experiments carried out in duplicate. Because normal distribution of the data could not be assumed, nonparamtric tests were performed to analyze the data. Statistical significance was determined by Mann-Whitney test for unpaired data or by Wilcoxon signed ranks test for paired data. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Keratinocyte Culture

Approximately 1×10^6 keratinocytes were isolated per 1 cm^2 harvested skin. Cells were seeded onto tissue culture plastic alone or on tissue culture plastic coated with collagen type IV. The appearance of keratinocytes in passage 2 (P2), P4, and P6 is illustrated in Figure 1. In P2, no difference in morphology was found between keratinocytes cultured with or without collagen type IV coating. The keratinocytes were polygonal in shape, with round, centered nuclei (Fig. 1A, B). In P4, keratinocytes cultured on plastic alone formed small colonies and exhibited a large, irregular shape. The morphology was less organized and cell boundaries were not clearly visible (Fig. 1D). In contrast, P4 keratinocytes cultured on collagen type IV still formed large colonies and exhibited a cubical shape (Fig. 1C). Keratinocytes grown on plastic alone reached senescence before P6, whereas all keratinocyte cultures grown on collagen type IV could be expanded at least to P6, although by this time the keratinocytes had an elongated and flattened appearance (Fig. 1E).

Table 1. Antibodies Used in the Immunohistochemical Analyses

Primary Antibody	Dilution	Source	Staining Normal Skin	Staining During Wound Healing	Reference
Keratin 17	1:100	Dako, Glostrup, Denmark	–	+	22
SKALP	1:1600	Hycult Biotechnology, Uden, The Netherlands	–	+	42
Involucrin	1:1000	Novocastra, Newcastle Upon Tyne, UK	+	+	8
Ki-67	1:50	Lab Vision, Fremont, USA	+	+	41
p63	1:50	Dako, Glostrup, Denmark	+	+	22
β_1-Integrin	1:1000	Calbiochem, San Diego, USA	+	+	15,45

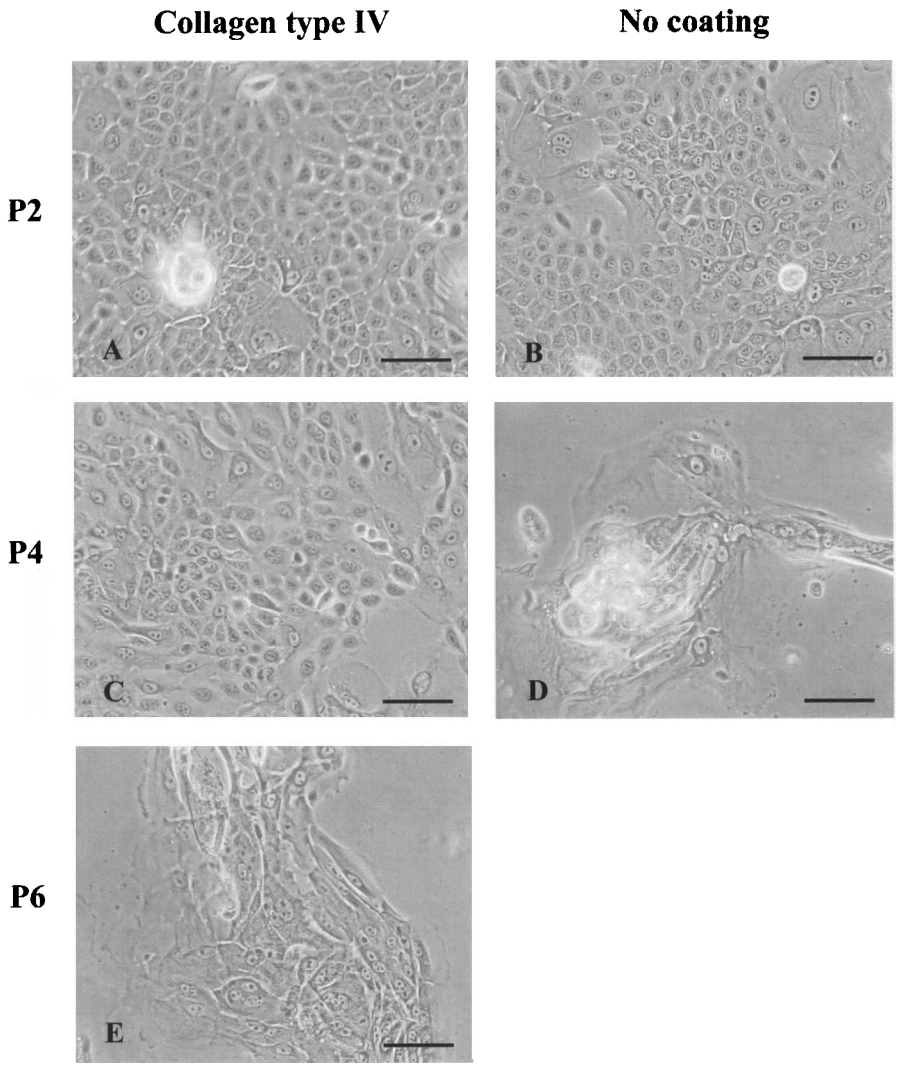

Figure 1. Phase contrast photomicrographs showing keratinocytes at different passage numbers. (A, C, E) Keratinocytes cultured on collagen type IV coating in passage 2 (P2), in P4, and in P6. (B, D) Keratinocytes cultured without collagen type IV coating in P2 and in P4. Original magnification: 100×, scale bars: 100 μm.

The passage number, in which the cells grown on collagen type IV undergo growth arrest, varied between different donors (P6–P10). In one occasion we were able culture keratinocytes until P20 (results not shown).

Up to P2, keratinocytes cultured with or without collagen type IV coating were passaged approximately every 4 days. After P2, keratinocytes cultured on collagen type IV grew faster than keratinocytes cultured on plastic alone (Fig. 2A). For this reason keratinocytes grown on coating were trypsinized every 4 days, whereas keratinocytes grown on plastic were trypsinized every 7 days after P2. The median culture time to reach P4 was 15 days for keratinocytes cultured with collagen type IV coating and 20 days for keratinocytes cultured on plastic alone ($p < 0.05$, Wilcoxon signed ranks test). The median amplification of keratinocytes cultured on collagen type IV was higher in P4 than the median amplification of keratinocytes cultured on plastic (19 and 7, respectively), but this result was not significant ($p > 0.05$, Wilcoxon signed ranks test) (Fig. 2B). In P2, no significant difference in amplification was found between keratinocytes cultured with or without coating ($p > 0.05$, Wilcoxon signed ranks test).

Morphology of the Reconstructed Epidermis

To examine the ability of the cells to reconstruct an epidermis, skin equivalents were made, using keratinocytes directly after isolation (P0), in P2, P4, and P6. All experiments were performed at least in twofold with keratinocytes obtained from three different donors. Skin equivalents made with P0, P2, and P4 keratinocytes cultured on collagen type IV showed a multilayered epidermis, which closely resembles normal skin (Fig. 3A, B, C, E). The reconstructed epidermis consisted of 8–9 viable cell layers and contained a well-defined basal layer, stratum spinosum, and stratum granulosum. The constructs contained a thicker epidermis than normal skin. P2 keratinocytes cultured without coating showed the same results (Fig. 3D). In contrast, using P4 keratinocytes cultured without coating, the formed epidermis was less organized and consisted of fewer cell layers (Fig. 3F). Keratinocytes in P6 (cultured on collagen type IV coating) formed a poor epidermal structure with very few viable cell layers (Fig. 3G).

Characterization of the Reconstructed Epidermis

To examine the reconstructed epidermis and to compare it to normal skin, expression of Ki-67, p63, and β_1-integrin was determined.

Ki-67-positive cells were located mainly in the basal layer of both reconstructed skin equivalents and normal skin (Fig. 4A). To determine the proliferation index, the percentage of Ki-67-positive cells in the basal layer was determined. Figure 4B shows that the median Ki-67 proliferation index (PI) was nearly the same in all skin equivalents. In contrast, the PI of normal skin (13%, range 11–16%) was lower than the PI of reconstructed skin equivalents, but this result was not significant ($p > 0.05$, Mann-Whitney test). Skin equivalents made using P4 uncoated and P6 coated keratinocytes were excluded from PI analysis, because these skin equivalents did not have a well-defined basal cell layer.

Figure 5A shows the expression of p63 in normal skin and in the reconstructed epidermis, made using ker-

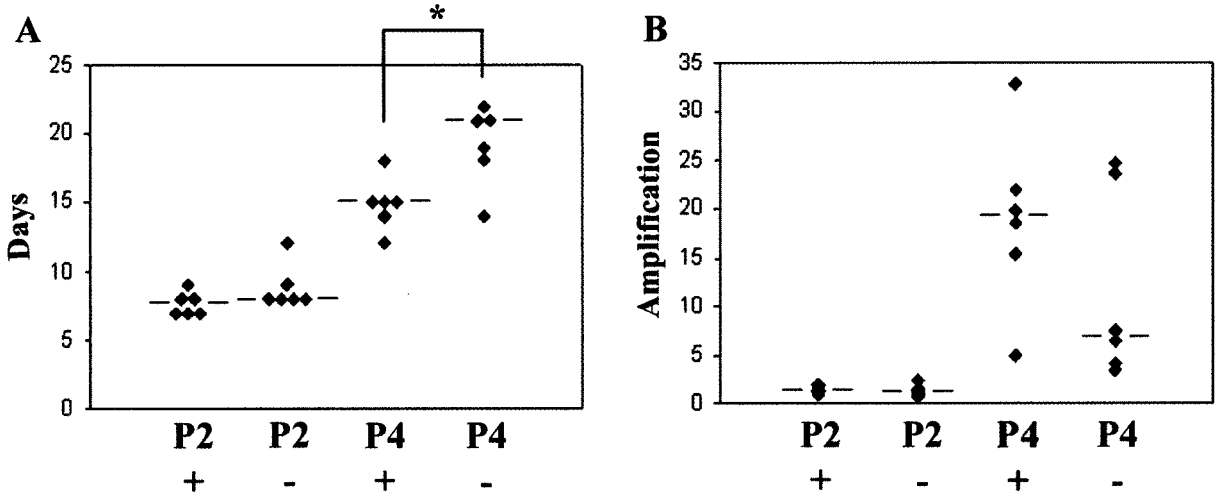

Figure 2. Culture time and amplification of cultured keratinocytes. (A) Number of days until keratinocytes that were cultured with (+) or without (–) collagen type IV coating reach passage 2 (P2) and P4. (B) Amplification of keratinocytes in P2 and P4 cultured with (+) or without (–) collagen type IV coating. The amplification factor was defined as the number of harvested keratinocytes divided by the number of seeded keratinocytes. The short thin lines indicate the median of the observations in each group. Statistical significance was determined by Wilcoxon signed ranks test. *$p < 0.05$.

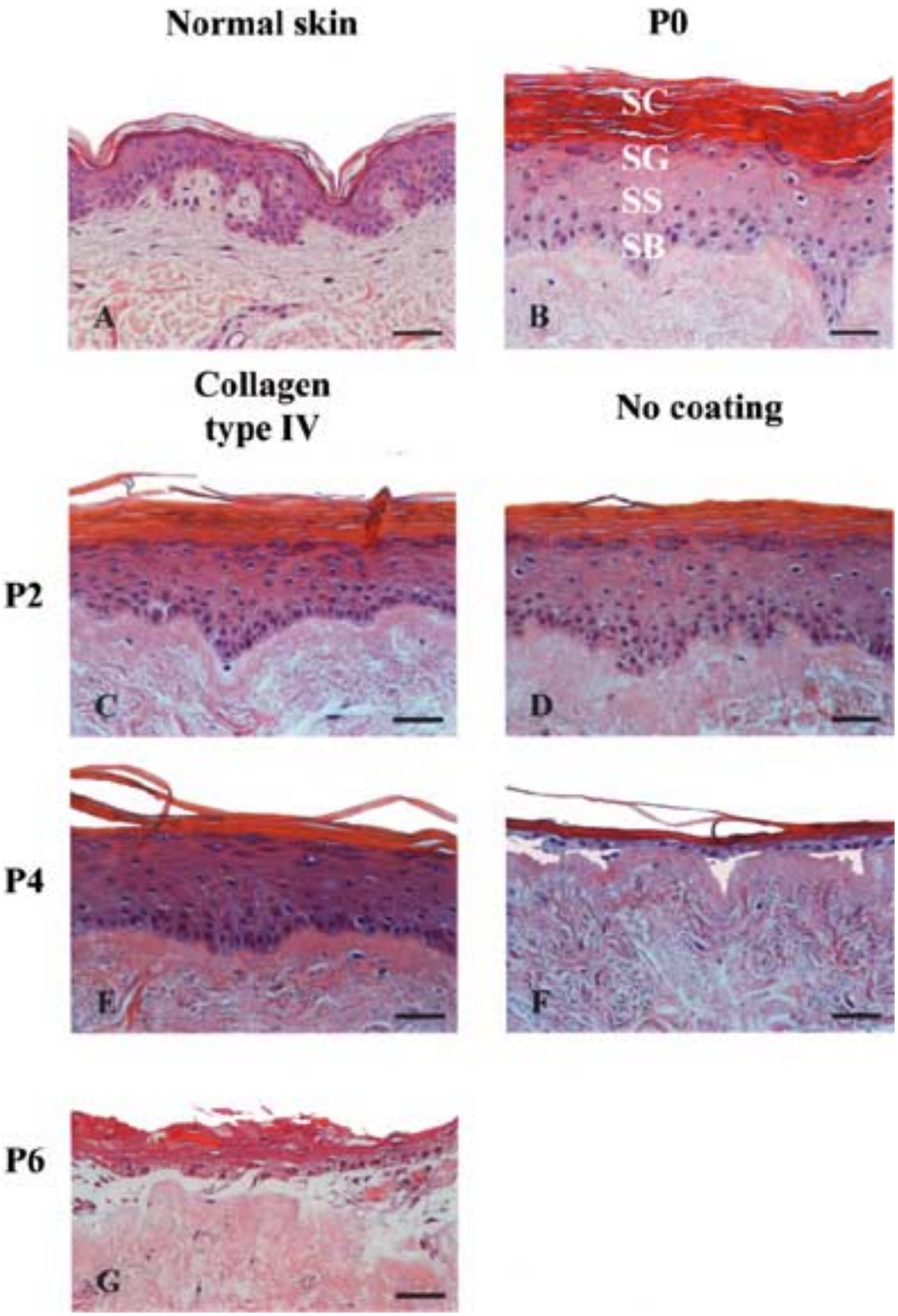

Figure 3. Histology of the reconstructed epidermis. Hematoxylin and eosin-stained sections of (A) normal skin and the skin equivalents made using (B) freshly isolated keratinocytes (P0), (C) keratinocytes cultured on collagen type IV coating in P2, (E) in P4, (G) in P6, (D) keratinocytes cultured without collagen type IV coating in P2 and (F) in P4. The differentiated epidermis contained a stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG), and stratum corneum (SC). All H&E sections were from one representative donor. Original magnification: 200×, scale bars: 50 μm.

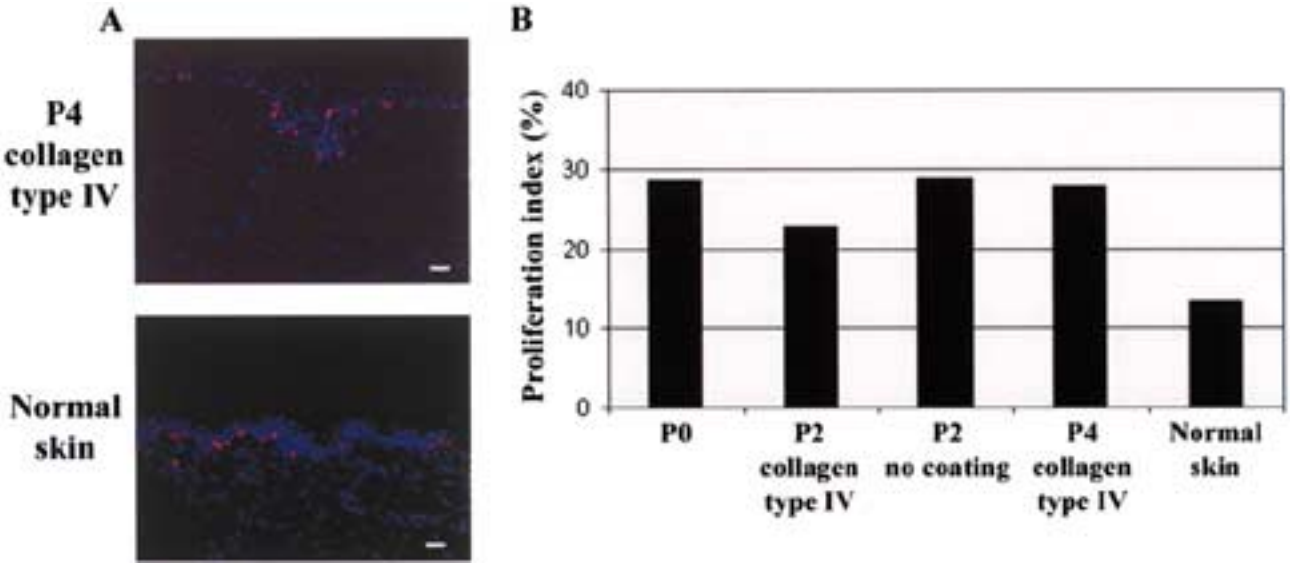

Figure 4. Expression of Ki-67 in normal skin and in skin equivalents. (A) The localization of Ki-67 in epidermis using keratinocytes in P4 cultured on collagen type IV and in normal skin is shown. Magnification: 100×, scale bars: 50 mm. (B) Proliferation index in reconstructed skin equivalents and in normal skin. The proliferation index was defined as the number of Ki-67-positive basal cells divided by the total number of basal cells × 100%. Each bar represents the median of three independent donors. Statistical significance was determined by Mann-Whitney test.

atinocytes cultured on collagen type IV. In both normal skin and cultured skin substitutes, p63 was present in the basal layer and in the lower layers of the stratum spinosum. The expression gradually decreased in the middle layer of the epidermis and no expression was seen in the stratum granulosum. The location of p63 expression was identical in all skin substitutes made using different passage numbers. No difference in expression was found between skin equivalents made using keratinocytes cultured on collagen type IV coating and skin equivalents made using keratinocytes cultured on plastic (data not shown).

β_1-Integrin expression in reconstructed epidermis and in normal skin is shown in Figure 5B. In epidermis constructed from freshly isolated keratinocytes, the expression of β_1-integrin was confined to the basal layer. In epidermis made with P2 and P4 keratinocytes, β_1-integrin was also present in suprabasal cell layers. The intensity of β_1-integrin staining gradually decreased towards the stratum granulosum. In normal skin β_1-integrin expression was only detected in the basal layer. No difference in expression was found between skin equivalents made using keratinocytes cultured on collagen type IV coating and skin equivalents made using keratinocytes cultured on plastic (data not shown).

Expression of Stress Markers in the Reconstructed Epidermis

To examine whether expression of stress markers differs in the cultured skin equivalents, the tissue specimens were stained with antibodies directed against SKALP, involucrin, K17. SKALP was not expressed in normal skin, but showed regular expression in all reconstructed skin equivalents (Fig. 6A). Expression of SKALP was detected in the stratum granulosum and in the upper layers of the stratum spinosum. Involucrin was expressed in the stratum granulosum in normal skin, but in all reconstructed skin equivalents involucrin was expressed in all suprabasal layers (Fig. 6B).

Figure 6C shows that K17 was not observed in normal skin. In contrast, K17 was expressed intermittently in most of the cultured skin equivalents, but it varied between different donors (data not shown). All donors showed that in P4 more expression of K17 was observed in epidermis made using keratinocytes cultured on plastic than in epidermis made using keratinocytes cultured on collagen type IV.

Minimal Required Seeding Density

To assess the minimal number of keratinocytes needed to form an epidermis, decreasing numbers (15×10^4–1×10^4/cm^2) of cells were seeded onto the DED. Only keratinocytes cultured on collagen type IV coating were used in this experiment. Of the freshly isolated keratinocytes 15×10^4 cells/cm^2 were necessary to form a differentiated epidermis, whereas of the cultured keratinocytes lower numbers of keratinocytes were still able to form an epidermis (Fig. 7). Of the P4 cells, 5×10^4 cells/cm^2 were sufficient to form an epidermis. P2 keratino-

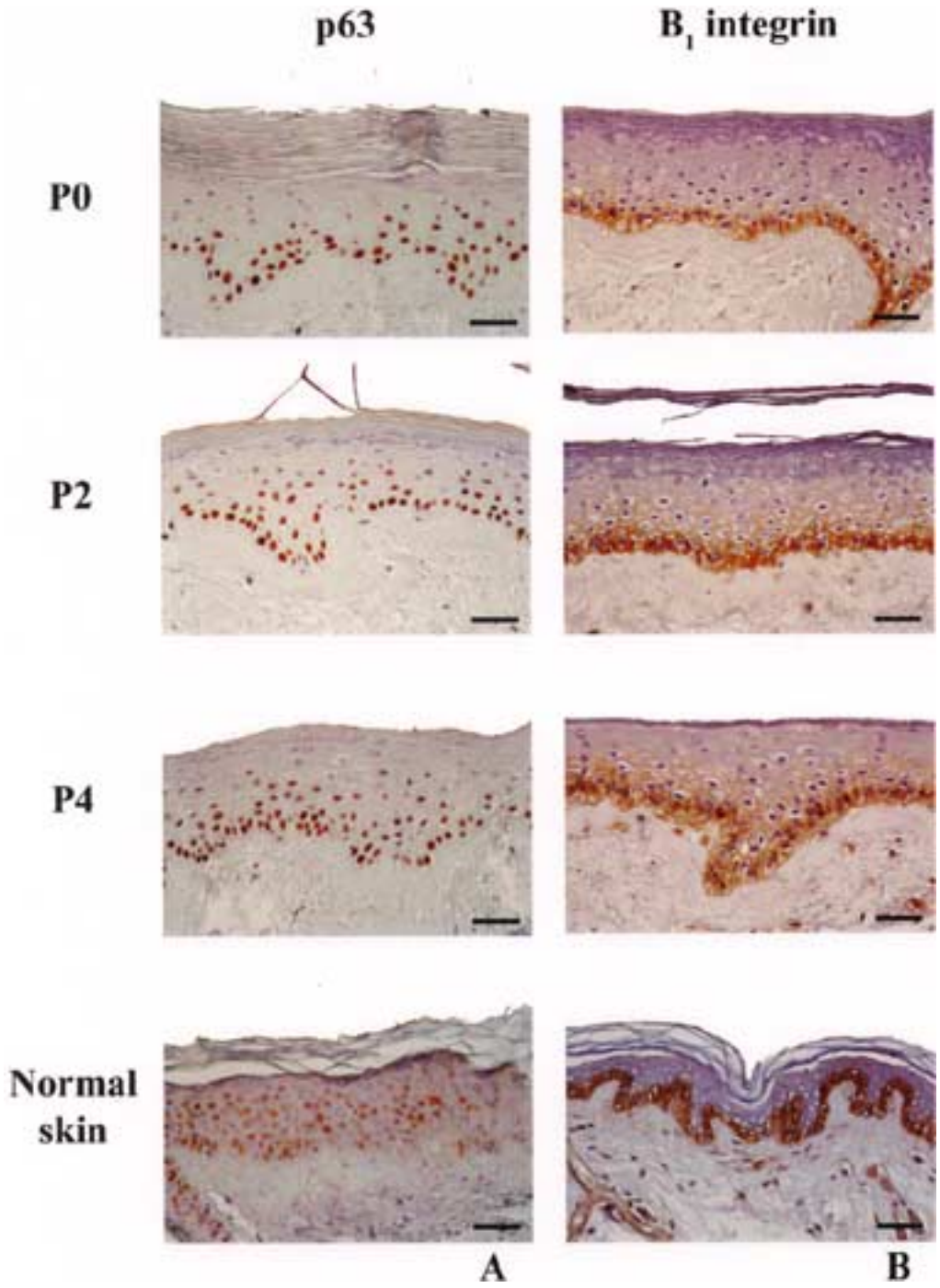

Figure 5. Expression of (A) p63 and (B) the integrin subunit β_1 in the reconstructed skin equivalents and in normal skin. Skin equivalents were made using freshly isolated keratinocytes (P0) or keratinocytes cultured on collagen type IV coating in P2 and P4. Original magnification: 200×, scale bars: 50 μm.

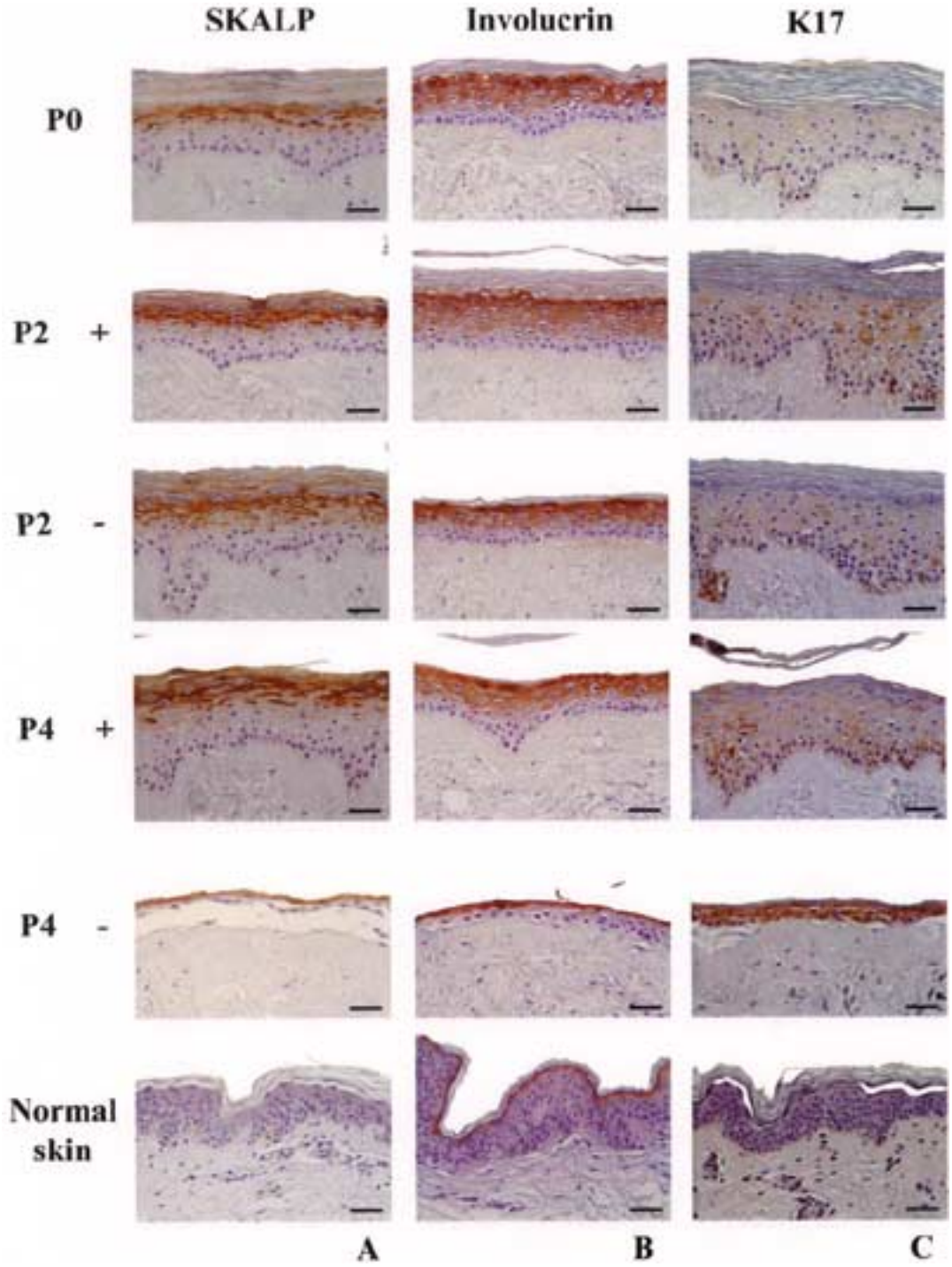

Figure 6. Expression of (A) SKALP, (B) involucrin, and (C) K17 is shown in the reconstructed skin equivalents and in normal skin. Skin equivalents were made using freshly isolated keratinocytes (P0) or keratinocytes in P2 and P4 cultured with (+) or without (–) collagen type IV coating. Original magnification: 200×, scale bars: 50 μm.

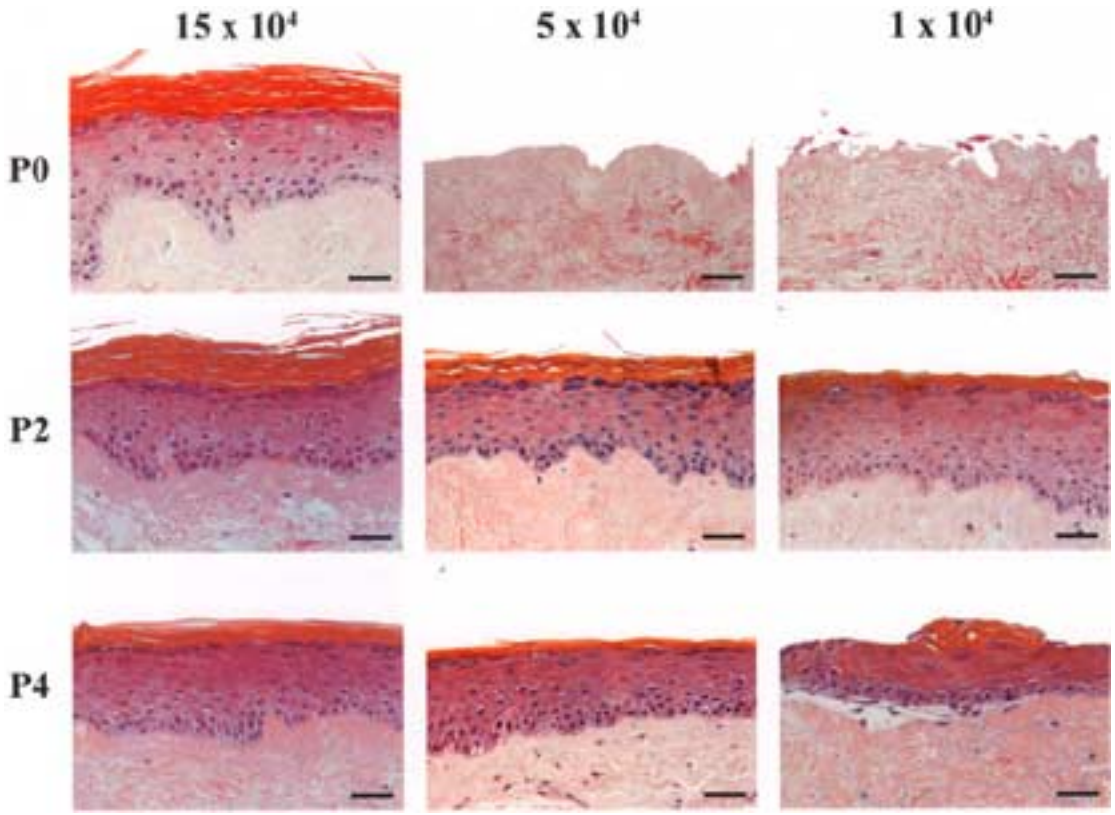

Figure 7. Histology of the skin equivalents made using decreasing numbers of keratinocytes. Hematoxylin and eosin-stained sections of the skin equivalents made using freshly isolated keratinocytes (P0) or keratinocytes cultured on collagen type IV coating in P2 and P4 in a density of 150,000, 50,000, and 10,000 cells/cm^2. All H&E sections were from one representative donor. Original magnification: 200×, scale bars: 50 μm.

cytes were still able to form an epidermis when only 1 $\times 10^4$ cells/cm^2 were seeded onto DED.

DISCUSSION

In the present study we show that keratinocytes can be cultured without the need of a fibroblast feeder layer and FCS. We show that these keratinocytes, when cultured on collagen type IV, are still able to generate a fully differentiated epidermis at P4.

Culture of keratinocytes without feeder layers and FCS has the advantage that it avoids the risk of transmitting infectious agents from animal origin and it simplifies culture procedures. In addition, European legislation would favor approval of xenobiotic-free materials above xenobiotic-containing materials for clinical use. Besides, it is feasible that in the future xenobiotic materials will be banned completely.

In this study we replaced FCS with Ultroser G. This serum substitute has been used in keratinocyte cultures with good results by ourselves and others (35). Although the composition of Ultroser G is semidefined, this study shows that it is possible to culture keratinocytes without FCS. Future efforts should be made to develop a completely defined medium for keratinocytes.

Epidermal reconstruction is a good way to examine the usefulness of keratinocytes for clinical application. Skin equivalents made using cultured keratinocytes showed a well-organized epidermis at least up to P4, when keratinocytes were cultured on collagen type IV. However, patient-to-patient variations were observed. In one occasion the keratinocytes could be cultured until passage 20 (results not shown).

An amount of 1 cm^2 harvested skin yielded 1×10^6 primary keratinocytes. A 19-fold amplification was achieved by culturing the keratinocytes on collagen type IV in 15 days. Because 50,000 cultured keratinocytes/cm^2 are sufficient to form an epidermis in P4, it is possible to obtain sufficient numbers of keratinocytes from 1 cm^2 skin, to potentially cover 400 cm^2 of wound surface. In comparison with the traditionally used 1:3 meshed split skin graft, which has an expansion rate lower than 3 (21), we achieved more than a 100-fold increase in wound covering over this gold standard technique.

In the reconstructed skin equivalents we found in-

creased expression of SKALP, Ki-67, and involucrin compared to normal skin. These markers are also increased in wound healing or psoriasis in vivo, and this suggests that the newly formed epidermis is in an activated state (8,41,42). Other studies also show that reconstructed skin equivalents display a hyperproliferative phenotype, similar to wounded skin and psoriatic epidermis (7,37). This implies that in cultured skin equivalents homeostasis is not (yet) established (7). Epithelial–mesenchymal interactions probably play an important role in maintaining homeostasis. Cultured skin equivalents show decreased expression of keratinocyte activation markers (K6, K16, and K17), if the skin equivalents are cultured with increased numbers of fibroblasts (6). In addition, supplementation of KGF, a growth factor produced by fibroblasts, decreases K6, K16, SKALP, and involucrin expression in skin equivalents (9). If the cultured skin equivalents were grafted in vivo the hyperproliferative phenotype is normalized, possibly because homeostasis is reestablished (38).

β_1-Integrins play an important role in keratinocyte adhesion and migration and are highly expressed on the surface of epidermal stem cells (45). Keratinocytes that express high levels of β_1-integrins are able to adhere rapidly to collagen type IV and form large, actively growing colonies (18). In the present study we found suprabasal expression of β_1-integrins in P2 and P4 cultured skin equivalents. This also indicates that the cultured skin equivalents are activated, because suprabasal integrin expression is observed during wound healing and in psoriatic lesions (15). However, no suprabasal integrin expression was found in epidermis made using freshly isolated keratinocytes. It has been suggested that in reconstructed skin equivalents suprabasal integrin is correlated to hyperproliferation (34). However, we did not find a lower expression of Ki-67 in skin equivalents made using freshly isolated keratinocytes. When keratinocytes are cultured in vitro, the cell surface of β_1-integrin increases (20). Therefore, cultured cells already had a higher expression of β_1-integrin than freshly isolated keratinocytes before skin equivalents were made. This may explain the difference in β_1-integrin expression seen in skin equivalents made using freshly isolated and cultured cells.

p63 is regarded as another potential epidermal stem cell marker based on the finding that $p63^{-/-}$ mice have severe defects in the development of stratified squamous epithelia (29,46). Increased expression of p63 is observed during wound healing (22). We found high expression of p63 in both normal skin and reconstructed skin equivalents. Other studies also show high expression of p63 in the basal and suprabasal layers of normal skin (25,27). Localization of p63-positive cells is associated with proliferative compartments in the epithelia (13). It is possible that p63 may play a role in maintaining proliferative potential of keratinocytes and preventing terminal differentiation (27).

Keratinocytes grown on collagen type IV-coated plates in P4 were still able to reconstruct an epidermis when only 50,000 cells/cm^2 were seeded, whereas 150,000/cm^2 of freshly isolated keratinocytes (P0) were necessary. Butler et al. also showed that cultured keratinocytes can more effectively form an epidermis than freshly isolated keratinocytes (2). They showed that cultured keratinocytes, grafted onto full-thickness wounds on Yorkshire pigs, were able to form a thicker epidermis with more subepithelial keratinocyte cysts per centimeter than uncultured keratinocytes. A likely explanation for this finding is that uncultured keratinocytes contain cells from all the different epidermal layers of the epidermis and contain only a small fraction of proliferating cells. During culture the proportion of proliferating cells increases, because differentiated cells do not adhere to culture flasks.

The results of the present study indicate that human keratinocytes can be cultured successfully without the use of feeder layer cells and FCS. When cultured on collagen type IV these cells are still able to form a fully differentiated epidermis up to P4. This culture technique can be a valuable tool for the further development of tissue engineered skin that can be used for treatment of burn wounds and other large and/or deep skin defects.

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