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Large surface of cultured human epithelium obtained on a dermal matrix based on live fibroblast-containing fibrin gels

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Abstract

The aim of this study was to develop a new keratinocyte culture system on a dermal equivalent suitable for skin wound closure. Our dermal matrix is based on a fibrin gel from plasma cryoprecipitate containing live human fibroblast (from human foreskin). Keratinocytes obtained from primary culture according to the Rheinwald and Green method, were seeded on the gel at different seeding ratios. In all cases, the keratinocytes plated on the dermal equivalent grew to confluence and stratified epithelium was obtained within 10–15 days in culture. Early expression of basal membrane proteins was detected by immunostaining with laminin and type IV collagen antibodies. Cell proliferation was detected both in the epidermal layer and in the fibroblast embedded in the gel as assessed by BrdU incorporation. Detachment of composite cultures from dishes or flasks is a simple and quick procedure without the need for dispase treatment. Grafting of composite cultures to nude mice gave rise to an orderly stratified, orthokeratinized epithelium resembling human epidermis. A number of advantages including a large expansion factor without the need of 3T3 feeder layer, the availability of fibrin/plasma cryoprecipitate from blood banks and the versatile manipulation of composite cultures suggest that this system could be suitable for the definitive coverage of severely burned patients.

Keywords: Keratinocyte culture; Fibrin; Fibroblasts; Dermal equivalent

1. Introduction

Since the development of the method for growing epithelial sheets with the support of lethally irradiated 3T3 cells in submerged culture conditions in 1975 [1], cultured epithelium has been used as grafting material in clinical situations such as the treatment of burn wounds [2], chronic skin ulcers [3] and oral mucous defects [4]. Particular attention received this methodology in the treatment of seriously burned patients since it allows to obtain in a short period of time, a large epithelium surface enough to cover the needs of a patient bearing a great surface of the corporal body damaged [5]. However, keratinocyte grafts have been found to have several persistent problems: blistering and contracture due to the lack of dermis and abnormal ultrastructure of the dermo-epidermal junction early after grafting [6]. These drawbacks have limited their clinical application and provided further stimulus to the development of keratinocyte culture methods on surfaces that mimic the human dermis (dermal equivalent). Several dermal equivalents of diverse composition have been reported [7–9]. In addition to the dermal bed often damaged by the burn, dermal substitutes increase the possibility of graft take [10]. However, the keratinocyte expansion factor found under these conditions (i.e. the total surface of keratinocyte culture that can be obtained in a given period) is lower than that obtained by the traditional culture in the presence of 3T3 cells [11]. So far, this restriction and the elevated cost of materials precluded the use of dermal equivalents for seriously burned patients, who require

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a large skin surface within a 3–4 week period. Data from several laboratories demonstrated the possibility of growing fibroblasts and endothelial cells on/in fibrin gels [12, 13]. Moreover, autologous keratinocytes in fibrin glues suspension have been employed in the treatment of burned patients [14, 15] and acellular fibrin gels have been used as biological support for keratinocyte cultures [16]. Based on these reports, we investigated the possibility that fibrin gels enriched with nonirradiated fibroblasts could act as a dermal substitute for keratinocyte culture. Bearing in mind the possible use of this type of culture for the treatment of seriously burned patients, we have evaluated our system in terms of cell proliferation, expansion rate, transplantability (onto nude mice) and handling of the composite culture for grafting purposes.

2. Material and methods

2.1. Primary keratinocyte culture

To obtain primary keratinocytes, normal human keratinocytes derived from human infant foreskin or adult skin donors were isolated following previously described methods [5]. 2×10^6 cells obtained were cultivated with 2×10^6 lethally irradiated 3T3–J2 (originally provided by Dr. H. Green, Harvard Medical School, Boston) or 5×10^6 Swiss albino mouse 3T3 (European Collection of Animal Cell Culture 85022108) in 75 cm^2 culture flasks. The culture medium was a 3:1 mixture of Dulbecco's modified Eagle medium (DMEM, Biochrom KG) and Ham-F12 (Biochrom KG) supplemented with 10% foetal calf serum (FCS) (Biochrom KG), insulin (5 μg/ml, Sigma), hydrocortisone (0.4 μg/ml, Sigma), triiodothyronine (1.3 ng/ml, Sigma), cholera toxin (8 ng/ml, Sigma) and adenine (24 μg/ml, Sigma). The medium was changed every three days, and at the end of the first change, epidermal growth factor (EGF) (10 ng/ml, Austral Biologicals) was added. This final medium will be referred to as keratinocyte culture medium (KCM). Cultures were incubated in a humidified 5% CO_2 atmosphere at 37°C. When keratinocyte primary cultures were preconfluent or at 24/48 h post confluence, they were treated with trypsin/EDTA to obtain individual cells. Then, they were counted, yielding $7–10 \times 10^6$ cells per flask, centrifuged and the pellet resuspended in 10 ml KCM. Cells were either subcultured on fibrin gels containing fibroblasts or frozen in DMEM containing 15% FCS and 10% glycerol (Sigma) for further use.

2.2. Preparation of fibrin and fibroblasts gels

Human fibroblasts were isolated from human infant foreskin or normal adults skin by enzymatic digestion, and cultured in DMEM containing 10% FCS. They were cultured in the fibrin-containing gels between their 4th and 12th passage.

Fibrinogen from plasma cryoprecipitates of human blood donors was used as the source of fibrin. The cryoprecipitates were provided by the local blood bank (Banco de Sangre del Centro Comunitario de Transfusión, Principado de Asturias, Spain) and were obtained according to the standards of the American Association of Blood Banks [17]. Each unit of cryoprecipitate was heated at 37°C for 45–60 s, and before complete thawing, it was centrifuged at 3500 rpm for 15 min at 4°C. The supernatant was discarded, the pellet dissolved in 10 ml of saline, and finally, heated at 37°C to complete fibrinogen dissolution. Around 150–300 mg of fibrinogen were obtained from each unit of cryoprecipitate.

To produce a fibrin gel, 3 ml of the fibrinogen (cryoprecipitate) solution were added to 12 ml of DMEM 10% FCS which contained 500,000 human fibroblasts and 5000 U/TIU of bovine aprotinine (Trasylol™, Bayer). Immediately after, 1 ml Cl_2Ca 0.025 mM (Sigma) with 11 U of bovine thrombin (Sigma) was added. Finally, the mixture was seeded in a 75 cm^2 culture flask and allowed (about 45 min) to solidify at 37°C. The gel was covered with KCM without EGF and either used immediately or 24 h later.

2.3. Secondary culture of keratinocytes on gel of fibrin and fibroblasts

The primary keratinocytes, prepared as described above, were seeded in the absence of 3T3 cells, onto a fibrin–fibroblast gel using the same KCM. For most experiments, 1/10 of primary cells in a 75 cm^2 flask was seeded in a 75 cm^2 flask containing the fibrin–fibroblast gel (ratio 1/10), and this means 9000–14,000 keratinocytes/cm^2 gel. In other experiments, the ratio of secondary culture was 1/30 and 1/50. So that, 30 or 50 flasks of the secondary culture were derived from each 75 cm^2 primary keratinocyte culture (see Table 1). Keratinocyte growth was followed using an inverted microscope, and when confluence was reached, the gels were manually detached from the culture flask. The culture medium was discarded and the gel was fixed to a nonpetroleum gauze at a few points by an organic polymer glue suitable for clinical use (Histoacryl™, Braun) [18]. The complete detachment of the culture from the flask was achieved and confirmed by examining the flask surface using an inverted microscope.

Parallel secondary cultures were performed on gels of fibrin without fibroblasts, either in the presence of

Table 1
The different subculturing ratios used in fibrin–fibroblasts gels. In square brackets data obtained from gels without fibroblasts

Keratinocytes seeding ratio/passage ratio (cells/cm^2 gel)	Time of confluence (days)	Stratification at day 15
9000–14,000 (1/10)	8–10 [13]	+ + [–]
3000–4700 (1/30)	10–12 [–]	+ + [–]
1800–2800 (1/50)	14–15 [–]	+ /– [–]

3T3 cells or in their absence. Also, fibrin gels containing fibroblasts were cultured without keratinocytes.

2.4. Histology and immunostainings

The gels were fixed in 10% formaldehyde and embedded in paraffin. The sections were stained with hematoxylin–eosin. Immunohistochemical studies with monoclonal antibodies against type IV collagen (Dako), laminin (Sigma) and broad spectrum human keratins (Dako) were performed. A pankeratin antibody or a human anti keratin K10 specific monoclonal antibody (AE-2, ICN) were also used to stain some samples derived from graft specimens.

2.5. BrdU assay

DNA synthesizing cells were identified on/in fibrin gels after incorporation for 18 h of 5-bromodeoxyuridine (BrdU, Boehringer). Labelled nuclei were visualized in paraffin sections of gels, using a BrdU specific monoclonal antibody (Amersham, proliferation kit RP-20) following manufacturer instructions.

2.6. Graft on nude mice

After keratinocyte confluence, cultures on fibrin–fibroblasts gels were manually detached and grafted on the dorsal region of athymic mice following the methods previously described by Barrandon [19]. The grafts were harvested 20 days later. Tissue specimens were either formalin-fixed or snap frozen to perform histological examination or immunostaining, respectively.

3. Results

3.1. Fibrin/fibroblasts gels allow the clonal growth of keratinocytes in the absence of 3T3 feeder layers

To test for keratinocyte expansion capacity on fibrin/fibroblasts gels, first passage keratinocytes obtained through the standard Rheinwald and Green culture [1] were seeded at different densities on fibrin gels containing 33,000 human fibroblasts/ml. Cell confluence occurred at all seeding densities tested, and a stratified epithelium was evident at 1/10 and 1/30 seeding ratio. At 1/50 passage ratio (1800–2700 keratinocytes/cm^2 density) a confluent epithelium bilayered in some regions was achieved at 14–15 days of culture (Table 1). In parallel cultures performed in the absence of human fibroblasts, confluence was delayed and only a nonstratified epithelium was reached at the highest keratinocyte seeding ratio (Table 1). In this case (i.e. without human fibroblasts), a confluent and stratified epithelium was only obtained when 3T3 cells were present.

3.2. Morphological and differentiation features of keratinocytes growing on fibrin–fibroblasts gels

At low seeding densities, keratinocytes grew in a clonal way (Fig. 1), forming colonies with no apparent signs of differentiation (scarcely stratified) (Fig. 2). From these colonies, the keratinocytes progressively occupy the entire flask surface until confluence to become afterwards a stratified epithelium (Table 1 and Fig. 3).

To assess the formation of basal membrane, type IV collagen and laminin expression were determined by immunohistochemistry using specific monoclonal antibodies (Fig. 4). Independently from the seeding density used, staining for both proteins was mainly detected at the dermo-epidermal boundary once confluence was reached. In parallel cultures performed in the absence of human fibroblasts, no staining for type IV collagen was observed and only a weak staining for laminin was present at day 15. In fibrin/fibroblasts gels without keratinocytes only a very weak staining for laminin was observed in fibroblasts.

After detachment, histological examination of the gel showed that keratinocytes were restricted to the external surface of the fibrin gel while fibroblasts to the inside, resulting in a composite material which maintains features of normal skin (Fig. 5).

Regardless of the expansion factor used, no retraction of the gels was observed throughout culture, although a slight thinning of the gel was observed from day 10.

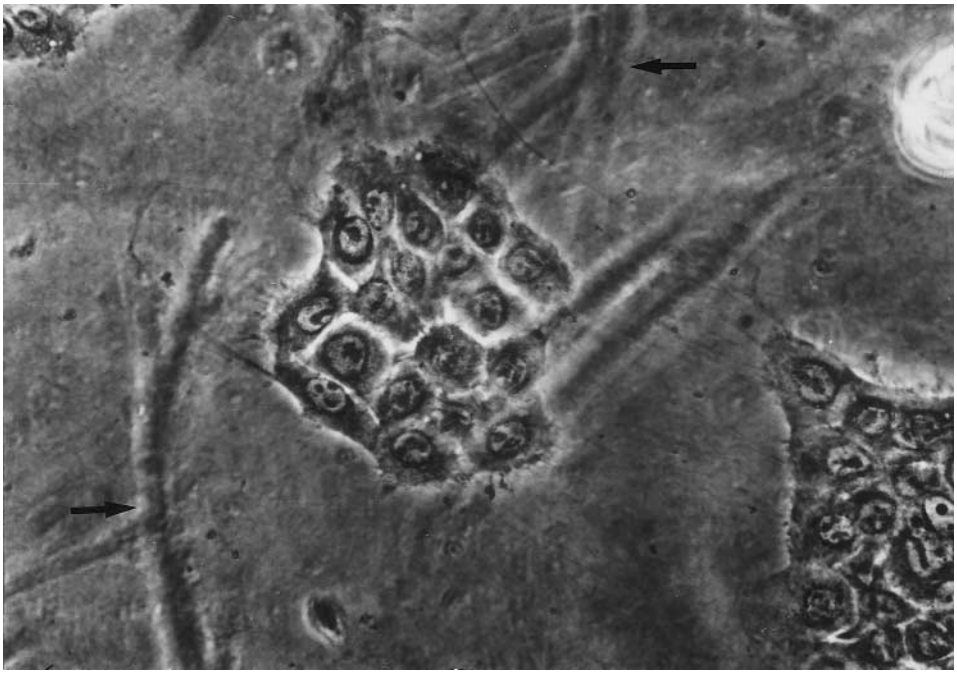

Fig. 1. Microscopic appearance (×200) of a keratinocyte colony on a fibrin–fibroblasts gel 48 h after seeding. Arrows indicate the presence of fibroblasts immersed in gel.

3.3. Cell proliferation occurs in epidermal and dermal compartments

Both, keratinocytes at the surface and fibroblasts incorporated into the fibrin gel proliferate as demonstrated by BrdU labelling studies (Fig. 6). A large proportion of keratinocytes was able to enter the S-phase indicating a high proliferative capacity even when confluence and stratification was already reached. Although BrdU incorporation was found also in fibroblasts, visualization of labelled cells was difficult due to their uneven distribution in the gel.

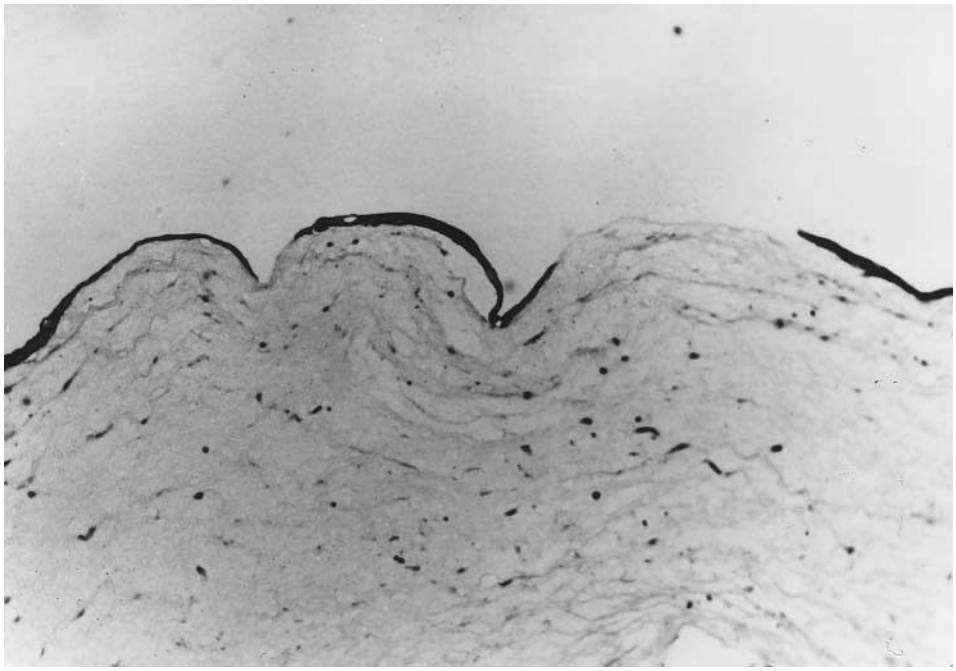

Fig. 2. Keratin immunohistochemical staining (×100) of keratinocytes cultured on fibrin–fibroblasts gels 4 days after seeding (seeding ratio 1/10). Three different not-stratified colonies can be seen.

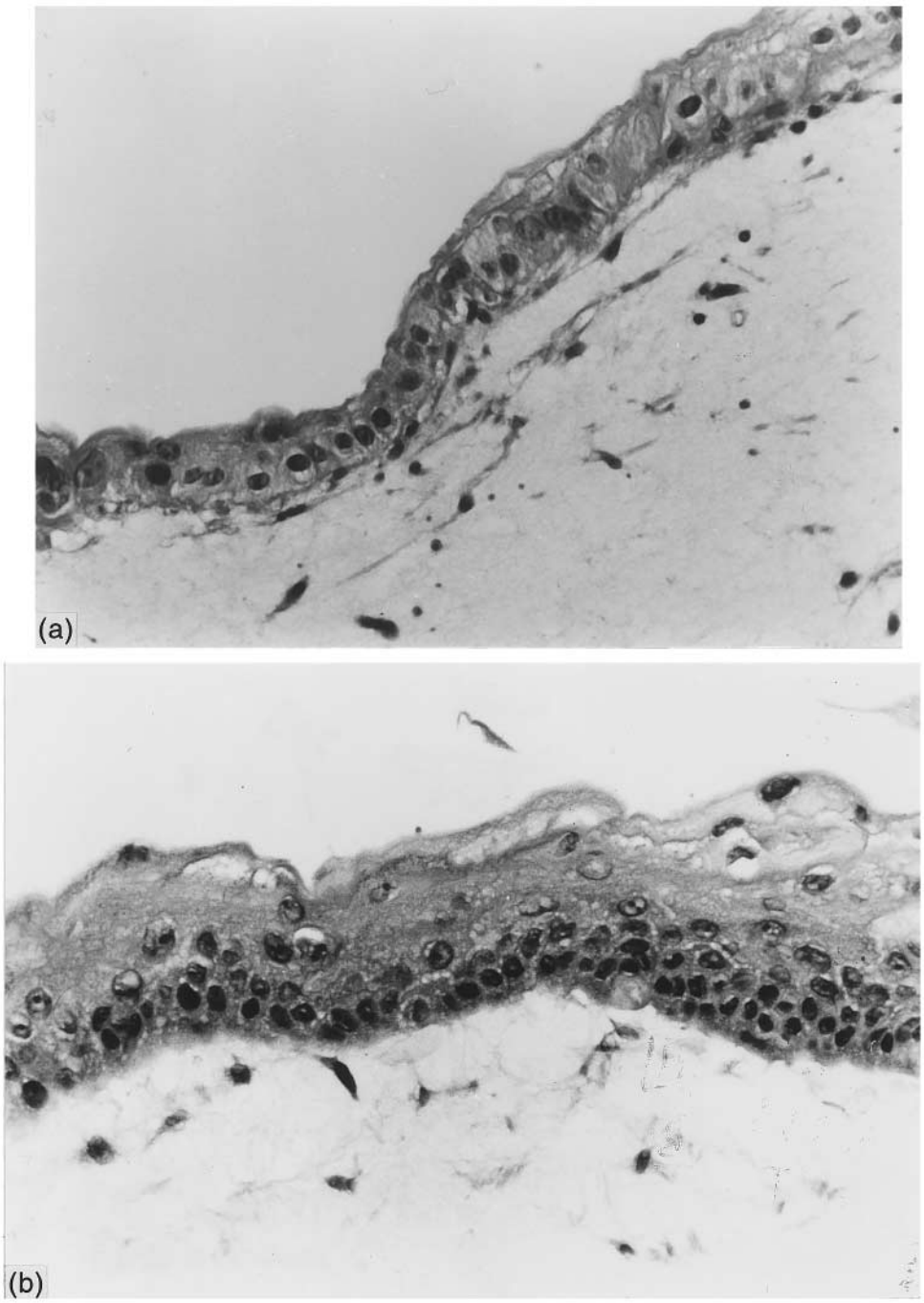

Fig. 3. Histologic appearance of keratinocytes cultured on fibrin–fibroblasts gel (H&E staining, ×250) 15 days after culture. Keratinocytes were seeded at 1/50 (a) and 1/30 (b) seeding ratio.

3.4. Grafting to athymic mouse

The grafts of composite cultures showed take in athymic mice. The macroscopic appearance of the grafted area was of a surface whiter than the surrounding rodent connective tissue. Histological studies showed the presence of a orthokeratinizated epithelium that was recognized by human antikeratin antibody (Fig. 7).

4. Discussion

This work shows that from a minimal skin biopsy and after a primary culture with 3T3 cells, the secondary culture on gels of fibrin and human fibroblasts permits the preparation of a large surface of stratified epithelium on a dermal layer equivalent. These confluent and stratified sheets are obtained even when keratinocytes were seeded at the lower expansion rate. At

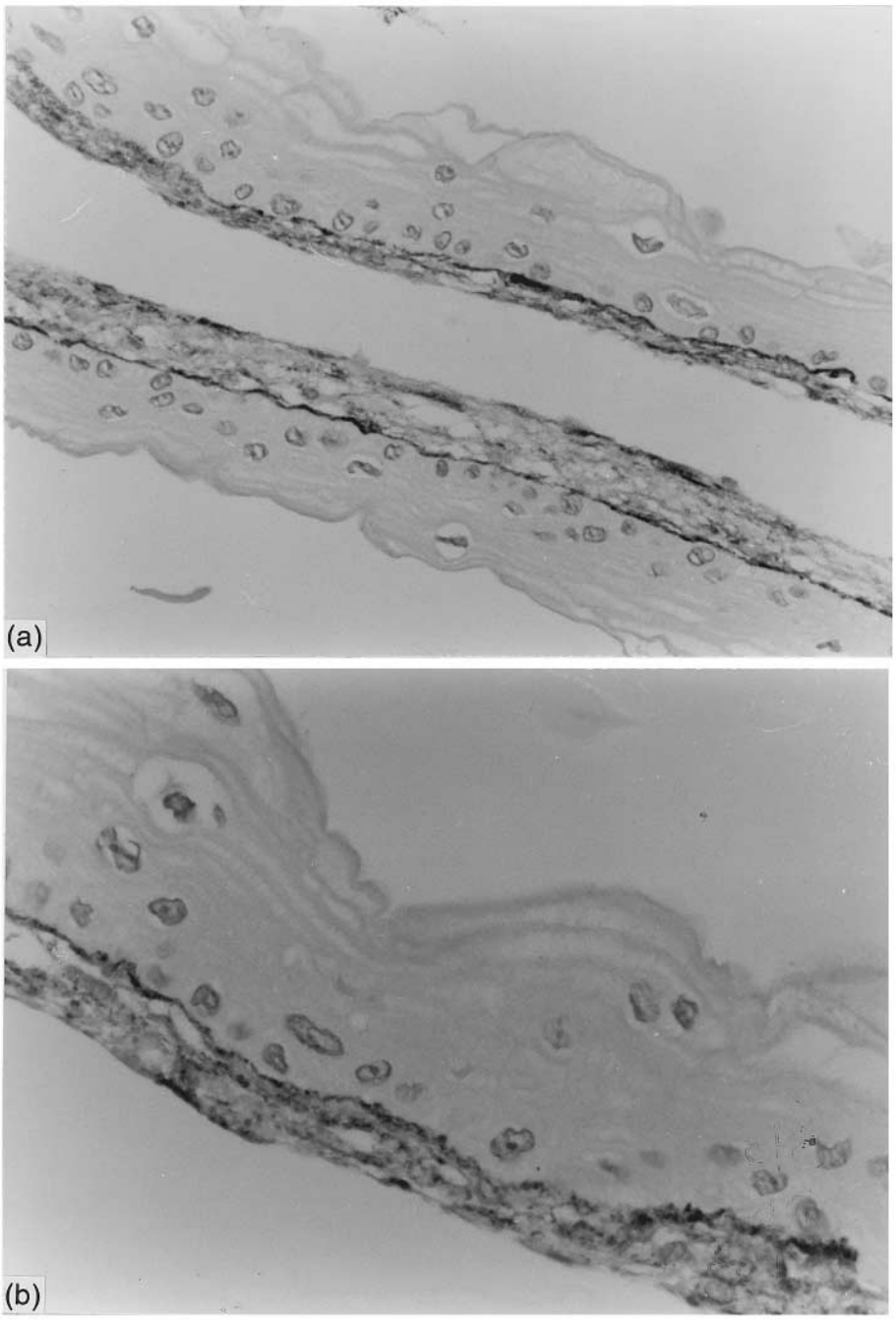

Fig. 4. Immunohistochemical staining of laminin (×250) (a) and collagen IV (×400) (b) in a confluent and stratified epithelium obtained by cultured keratinocytes on fibrin–fibroblasts gel (day 15 of culture).

this lower seeding ratio (1/50) more than 1 m^2 of secondary culture on gel can be obtained in about 25 days from a skin biopsy of 2–4 cm^2. This represents a very high expansion, similar to that obtained with the culture of keratinocytes in the presence of irradiated 3T3 cells, the most widely used culture system for treatment of severely burned patients. In this composite culture there is an early expression of membrane proteins, such as, type IV collagen and laminin and finally, this culture system is able to take in an experimental model of grafting in mice.

Several technologies are at the moment under active development as aids in cutaneous wound repair. However, many of them deal with a single aspect of the healing process that does not necessarily serve as primary source of new tissue. The biochemical and cellular composition of the composite graft described in this paper closely resembles that of skin during the

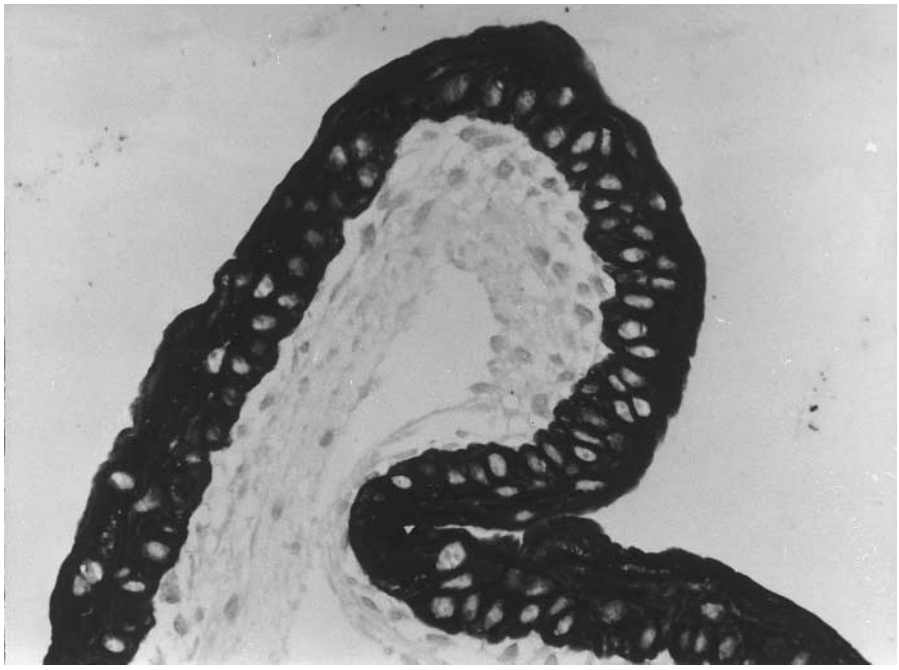

Fig. 5. Histological appearance of a keratinocyte cultured gel manually detached. Keratin immunohistochemical staining (×250) of human keratinocytes cultured on fibrin–fibroblasts gels 15 days after seeding (seeding ratio 1/10).

first steps of wound healing. In fact, during the healing of a skin wound, the defect is temporally plugged with the formation of a fibrin clot. This clot is later infiltrated by inflammatory cells, fibroblasts and granulation tissue [20]. Thus, a high efficacy for wound closure could be expected, since an already-accomplished step of healing is provided.

The fibrin gels have been prepared from fibrinogen obtained by cryoprecipitation of human plasma. The cryoprecipitate is a product normally used in haematological practice, and contains a large amount of fibrinogen, factor VIII, factor XIII and fibronectin. Although it is difficult to obtain a constant fibrinogen concentration from the cryoprecipitate (this varied

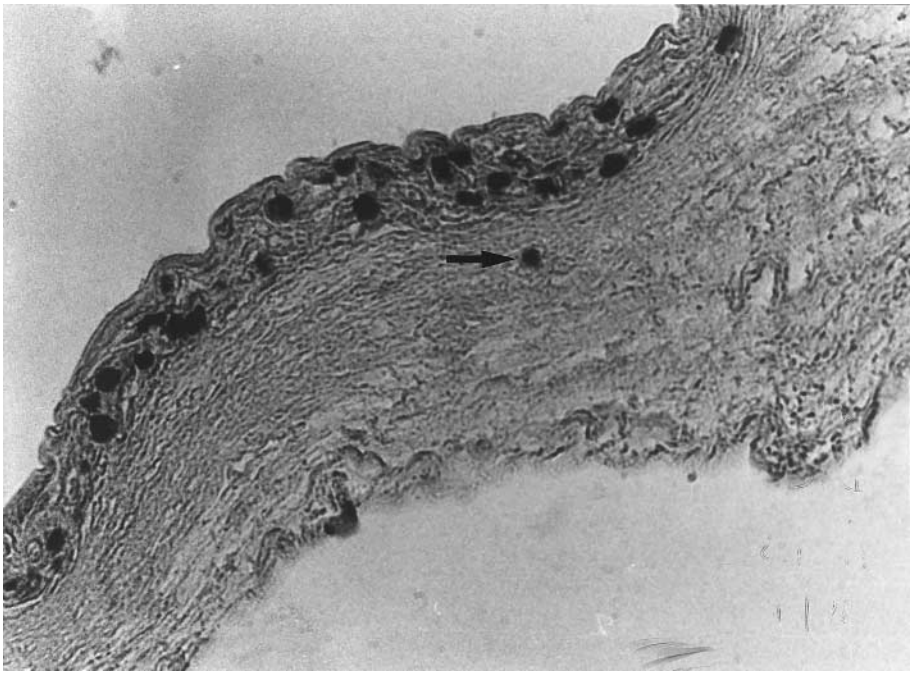

Fig. 6. BrdU incorporation to a keratinocyte cultured epithelium on fibrin–fibroblasts gel at confluence. Arrow indicates a labelled fibroblast nucleus.

between 4 and 9 mg/ml), no differences were observed in the gels when the fibrinogen concentration was kept within this limit. (The use of higher fibrinogen concentrations does not allow the observation of keratinocyte growth by microscope.) With lower concentrations of fibrinogen, some gels become dissolved before the

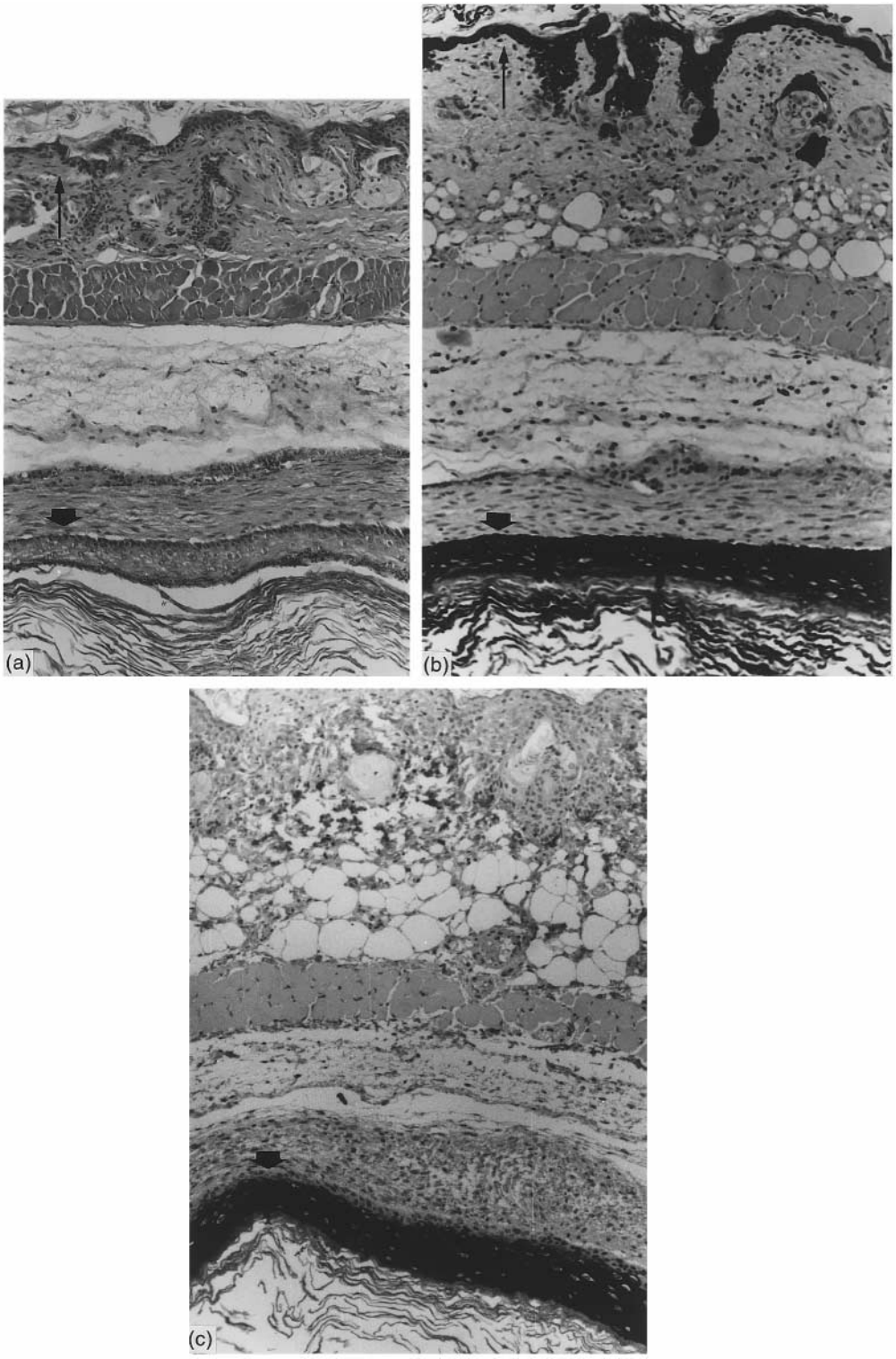

Fig. 7. Histological appearance of grafted cultured epithelium in the inner surface of the dorsal region in an athymic mouse. H&E stained section of a day 20 biopsy taken at graft site (×100) shows (a) a sandwich consisting of two epidermal coverings separated by connective tissue, the outer (top, ↑ thin arrows) consisting of rodent epidermis and the inner (bottom, ↓ thick arrows) the human epidermis. Immunohistochemical staining with a pankeratin antibody (b) and a specific human antikeratin antibody (c).

complete confluence of keratinocytes has occurred (data not shown). Fibrinogen is also available from commercial sources. The use of this commercial fibrinogen has not been tested for this study. Commercial fibrinogen is heat inactivated and this treatment could probably modify the presence of cytokines, growth factors and other adhesion molecules present in the cryoprecipitate product perhaps decreasing the high proliferative capacity of cells (shown by BrdU labelling).

Positive staining for two basal membrane proteins, type IV collagen and laminin were found in confluent cultures. The expression of these membrane proteins seems to rely on the presence of both keratinocytes and human fibroblasts in the culture. In fact, in those cultures done in the absence of either fibroblasts or keratinocytes, no expression of type IV collagen or only a slight expression of laminin can be detected (data not shown). The presence in the culture of basal membrane structures could favor the grafting success. In fact, when the composite cultures were grafted on athymic mice, they were able to take and develop an epidermis which resembles human epidermis with a cornified layer included.

The culture of keratinocytes on gels of fibrin and fibroblasts is easily handled. It can be performed in a closed culture flask with a lower risk of contamination than in a petri dish. Also, since the gel is transparent, the growth of the keratinocyte colonies can be followed with the inverted microscope. However, the greatest handling difference of this culture compared with traditional cultures is that sheets can be manually detached from the flask. By using this method of separation, the continuity of the layer of the keratinocytes as well as the integrity of their basal layer is preserved. There is no loss of surface area, such as occurs when the sheets of isolated epithelium are processed with dispase II and fixed to a gauze with ligaclips.

Finally, the last step of this composite culture, could be its grafting to patients. Regarding the possible clinical use of these fibrin–fibroblasts gels, it must be remarked that the use of fibrin has been successful for the treatment of burned patients, either to fix a skin graft [21, 22], cultured keratinocytes in suspension [14, 15] or cultured keratinocyte sheets [23, 24]. In all cases, fibrin did not interfere with the normal development of the definitive link between the graft and the wound bed. The number of fibroblasts expanded from a small skin biopsy during the 10–12 days of primary culture is lower than the number used in the present study and thus, autologous fibroblasts can not be used. Although it has been reported that fibroblasts are immunogenic [25], their possible involvement in the graft rejection has not been clearly established [26, 27]. Moreover, allodermis of cryopreserved skin with live dermal cells, which was shown not to be rejected, have been successfully used as dermal bed, and homologous fibroblasts are components of different dermal substitutes [28]. This led to the idea that skin equivalents may be constructed using allogeneic fibroblasts [29].

In conclusion, the culture of keratinocytes on gels of fibrin containing living fibroblasts offers some advantages on other previously reported methods such as: (1) high expansion factor; (2) easy monitoring of keratinocyte growth, handling and delivery for grafting; (3) elimination of enzymatic treatment and (4) application of epithelial and mesenchymal cells in a single operative procedure.

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