Abstract
Background. Cell therapy is an emerging therapeutic strategy aimed at replacing or repairing severely damaged tissues with cultured cells. Epidermal regeneration obtained with autologous cultured keratinocytes (cultured autografts) can be life-saving for patients suffering from massive full-thickness burns. However, the widespread use of cultured autografts has been hampered by poor clinical results that have been consistently reported by different burn units, even when cells were applied on properly prepared wound beds. This might arise from the depletion of epidermal stem cells (holoclones) in culture. Depletion of holoclones can occur because of (i) incorrect culture conditions, (ii) environmental damage of the exposed basal layer of cultured grafts, or (iii) use of new substrates or culture technologies not pretested for holoclone preservation.

The aim of this study was to show that, if new keratinocyte culture technologies and/or "delivery systems" are proposed, a careful evaluation of epidermal stem cell preservation is essential for the clinical performance of this life-saving technology.

Methods. Fibrin was chosen as a potential substrate for keratinocyte cultivation. Stem cells were monitored by clonal analysis using the culture system originally described by Rheinwald and Green as a reference. Massive full-thickness burns were treated with the composite allografts/cultured autograft technique.

Results. We show that: (i) the relative percentage of holoclones, meroclones, and paraclones is maintained when...
keratinocytes are cultivated on fibrin, proving that fibrin does not induce clonal conversion and consequent loss of epidermal stem cells; (ii) the clonogenic ability, growth rate, and long-term proliferative potential are not affected by the new culture system; (iii) when fibrin-cultured autografts bearing stem cells are applied on massive full-thickness burns, the "take" of keratinocytes is high, reproducible, and permanent; and (iv) fibrin allows a significant reduction of the cost of cultured autografts and eliminates problems related to their handling and transportation.

Conclusion. Our data demonstrate that: (i) cultured autografts bearing stem cells can indeed rapidly and permanently cover a large body surface; and (ii) fibrin is a suitable substrate for keratinocyte cultivation and transplantation. These data lend strength to the concept that the success of cell therapy at a clinical level requires cultivation and transplantation of stem cells. We therefore suggest that the proposal of a culture system aimed at the replacement of any severely damaged self-renewing tissue should be preceded by a careful evaluation of its stem cell population.

Lining epithelia are renewed constantly during the lifetime of an organism. For instance, human epidermis is replaced approximately every month (1). To accomplish their self-renewal process, lining epithelia rely on the presence of stem and transient amplifying cells, which are the only proliferative cells in a normal tissue (for review see 2-4). Stem cells can be defined as cells endowed with a high capacity for cell division and also able to generate a differentiated progeny (2, 4, 5). The extensive proliferative potential, at least maintained through the lifetime of an organism, is considered the basic and essential characteristic of a stem cell (4). Transient amplifying cells, which arise from stem cells, have a high proliferative rate only for a limited period of time and represent the largest group of dividing cells (2, 3, 5). Epidermal (and other lining epithelial) stem and transient amplifying cells can be identified in culture (1, 2, 6-13). Indeed, using clonal analysis, three types of keratinocytes with different capacities for multiplication have been characterized (6). The holoclone, which is generated by the epidermal stem cell (2, 6, 12, 13), has a tremendous potential for proliferative self-renewal, being able to undergo more than 140 doublings before senescence. The paraclone, which is generated by a transient amplifying cell, has a very limited growth potential (being committed to a maximum of 15 divisions) and usually gives rise to aborted colonies (6). The meroclone is an intermediate type of cell and is a reservoir of transient amplifying cells (2, 6). The transition from holoclone to meroclone to paraclone is an irreversible unidirectional process that occurs slowly during ageing (6).

Cell therapy is an emerging therapeutic strategy aimed at replacing or repairing severely damaged tissues with cultured cells (14). The choice of cells to be propagated in culture strictly depends on the function that is required of the cell after transplantation, to assure the persistence and function of the regenerated tissue through the life span of the patient. For instance, the complete replacement and continued turnover of the hematopoietic tissue requires the engraftment of bone marrow-derived hematopoietic progenitor cells (14). This concept holds true for tissues, such as lining epithelia, that experience a continuous self-renewal process during life (14-16).

This said, human epidermal keratinocytes, serially propagated in vitro (17), generate cohesive sheets of stratified epithelium, which maintains the characteristics of authentic epidermis (18-22). Autologous cultured keratinocytes (cultured autografts) have been widely used for the permanent coverage of massive full-thickness burns (for reviews, see 23-29 and references therein), and epidermal regeneration obtained with cultured autografts can be life-saving (24, 27, 30-33). Permanent "take" of cultured autografts was formally proven by re-expression of site-specific differentiation markers in vivo (34). By the early 1990s, however, the initial optimism for this technique declined, mainly because of the poor clinical results that have been consistently reported by different burn units (see 25, 28, 35, 36, and references therein). Thus, the real usefulness of cultured keratinocytes in massive full-thickness burns is currently questioned by several surgeons (see 25, 28, 35, 36 and references therein).

Control of infection and proper preparation of the wound bed allow a good "take down" of cultured autografts, that is, take of the cultures at the first clinical evaluation (7-10 days after grafting) (27, 35, 37-39). This has been demonstrated by the increased take down of cultured autografts on a wound bed prepared with allogeneic human dermis, a natural substrate for keratinocyte adhesion, growth, and proper differentiation (36-41).

However, the key aim of the clinical response to the treatment of full-thickness burns with cultured autografts is the ensuing permanent epidermal regeneration ("final take"). Because human epidermis is replaced monthly, the long-term persistence of the regenerated epidermis requires stem cell transplantation. Therefore, it is conceivable to speculate that the poor clinical performance of cultured autografts transplanted on a properly prepared wound bed (see 25, 28, 35, 36, and references therein) or the unexplained loss of the cultured autografts after an initial take down (29), might actually arise from depletion of stem cells in culture. Loss of epidermal holoclones in culture can be due to: (i) incorrect culture conditions (our unpublished data), (ii) environmental damage of the exposed basal layer of cultured grafts, or (iii) use of new substrates or culture technologies not pretested for stem cell preservation.

The latter is of particular importance because of: (i) surgical needs; (ii) the intrinsic high cost of keratinocyte cultures (especially if one relates the cost of the cultures to the percentage of the healed body surface) (27, 42, 43); (iii) the need for detachment of the epidermal sheets from the culture vessel (a time consuming and costly procedure that can cause damage to the stem-cell containing clonogenic basal layer and that reduces the size of the grafts); and, (iv)
problems in handling and long-distance transportation, which have prompted several laboratories to develop "simplified" culture media or to propose new culture technologies envisaging cultivation of epidermal cells onto different carriers (15,44). Maintenance of stem cells, however, has never been demonstrated in these new culture systems, sharpening the lack of trust to the technology.

This prompted us to investigate whether a careful control of stem cells in cultured autografts was followed by a good and reproducible clinical performance of keratinocyte cultures. Among the different keratinocyte "delivery systems" available (see Discussion), we have chosen the fibrin sealant (45, Ronfard and Barrandon, Ecole Normale Superieure, Paris, France, personal communication, 1995) because fibrin is a readily available natural substrate, is usually abundant in wound healing, and is quickly degraded by the host. We show that: (i) keratinocyte clonogenic ability, growth rate, and long-term proliferative potential are not affected by fibrin; (ii) human epidermal stem cells are preserved when keratinocytes are cultivated on a fibrin substrate; and (iii) clonal conversion is not accelerated by fibrin. These data fulfill the most important requirements for a keratinocyte culture to be used for the treatment of massive full-thickness burns. We also show that: (i) when fibrin-cultured autografts bearing stem cells are applied on massive full-thickness burns, the final take of keratinocytes is high (100%), reproducible, and permanent; and (ii) fibrin allowed us to obtain two- to threefold larger grafts (as compared with plastic) from the same number of clonogenic cells and eliminates the need for detachment of the epidermal sheet with a consequent significant reduction of costs and of problems in handling and transportation.

**Back to Top**

**MATERIALS AND METHODS**

The fibrin sealant. The fibrin sealant (Tissucol) was purchased from Baxter-Immuno (Wien, Austria). Tissucol is composed of two frozen fibrinogen and thrombin stock solutions. The fibrin gels were prepared as follows: solution A was obtained by diluting the original thrombin stock solution to a final concentration of 3 IU/ml thrombin in 1.1% NaCl and 1 mM CaCl₂. Solution B was prepared by mixing 5 ml of the original fibrinogen stock solution with 5.8 ml of a saline solution (final concentrations: 1.1% NaCl; 1 mM CaCl₂).

The fibrin gels were then prepared in 144-cm² plates not treated for cell culture (Greiner, Stuttgart, Germany). To obtain a 100-µm thick fibrin gel, 3.6 ml of solution A and 3.6 ml of solution B were uniformly mixed in each plate; the plates were left at room temperature for 10-15 min (until a complete substrate polymerization was obtained) and stored overnight at 4°C. Cell culture. 3T3-J2 cells were cultured in Dulbecco's-Vogt Eagle's medium (DMEM*) containing calf serum (10%), glutamine (4 mM), and penicillin-streptomycin (50 IU-50 µg/ml).

Human epidermal keratinocytes were cultured as previously described (12,17-19). Briefly, skin biopsy samples were minced and trypsinized (0.05% trypsin/0.01% EDTA) at 37°C for 3 hr. Cells were collected every 30 min, plated (2.5×10⁴/cm²) on lethally irradiated 3T3-J2 cells (2.4×10⁴/cm²), and cultured in 5% CO₂ and humidified atmosphere in keratinocyte growth medium: DMEM and Ham's F12 media (2:1 mixture) containing 10% fetal calf serum, insulin (5 µg/ml), adenosine (0.18 mM), hydrocortisone (0.04 µg/ml), cholera toxin (0.1 nM), triiodothyronine (2 nM), epidermal growth factor (10 ng/ml), insulin (4 mM), and penicillin-streptomycin (50 IU-50 µg/ml). Subconfluent primary cultures were serially propagated as described (12,19), either on plastic or on fibrin (prepared as above). Keratinocytes were cultivated in the presence of lethally irradiated 3T3-J2 cells and in keratinocyte growth medium, both on plastic and on fibrin and plated at a cell density of 5×10³/cm². Efficiency of colony formation (CFE) by keratinocytes was determined by plating 100-1000 cells, fixing colonies with 3.7% formaldehyde 9-14 days later, and staining them with 1% rhodamine B (6,12).

Fibrin-cultured autografts (subconfluent to confluent secondary cultures) ready for grafting were washed twice in DMEM containing 4 mM glutamine and penicillin-streptomycin (50 IU-50 µg/ml). Fibrin-cultured autografts were gently removed from the plates with the aid of two forceps and placed in sterile, biocompatible, and non-gas-permeable 144-cm² polystyrene boxes (see Fig. 1A) containing gauze. Boxes were closed, equilibrated at 37°C in 5% CO₂ humidified atmosphere for 15 min, thermo-sealed, and transferred to the burn unit.

Clonal analysis. For clonal analysis, keratinocytes cultivated for 7 days on plastic or on fibrin were trypsinized, and single cells, isolated under the microscope, were plated onto lethally irradiated 3T3-J2 cells in multwell plates. After 7 days of cultivation, plates were carefully scored for the presence of a colony under a microscope. Colonies were photographed under a Zeiss Axiovert microscope, and their areas were measured using a computerized image analyzing system. Each clone was then transferred by trypsinization to two indicator dishes. One dish was used for primary cultures were serially propagated as described (12,19) and then stained with rhodamine B for 9-14 days later, and the second dish was fixed and stained with 1% rhodamine B (6,12). Clonal analysis. For clonal analysis, keratinocytes cultivated for 7 days on plastic or on fibrin were trypsinized, and single cells, isolated under the microscope, were plated onto lethally irradiated 3T3-J2 cells in multiwell plates. After 7 days of cultivation, plates were carefully scored for the presence of a colony under a microscope. Colonies were photographed under a Zeiss Axiovert microscope, and their areas were measured using a computerized image analyzing system. Each clone was then transferred by trypsinization to two indicator dishes. One dish was used for primary cultures were serially propagated as described (12,19) and then stained with rhodamine B for 9-14 days later, and the second dish was fixed and stained with 1% rhodamine B (6,12).
Full-thickness burns covered 20% to 45% of the body surfaces of the patients (see Results). The patients’ ages ranged between 1.7 and 30 years (average 16.4 years). Full-thickness skin biopsy specimens (1-7.5 cm²) were taken within 2 to 13 days after admission, placed in keratinocyte growth medium (without epidermal growth factor), and transferred refrigerated to the laboratory within 16-20 h after drawing. Keratinocytes were cultured as described above, and fibrincultured autografts were prepared as above. The patients’ wound bed was prepared with allogermis as previously described (37-39). Briefly, fresh donor skin was harvested with a Padgett electric dermatome set at 0.70 mm; allografts were subsequently meshed 1:2. The burn eschars were tangentially excised, and excisions were made down to viable tissue. Immediately after escharectomy, the meshed donor skin was transplanted onto patients. Allografts were stapled in place by metallic clips and overlaid with Vaseline gauzes followed by dry gauzes on the top. Dressing changes were done every 2-3 days under surgical management. Documented infections were successfully treated by topical and/or systemic treatment.

Approximately 2 weeks later, when fibrin-cultured autografts were ready, the allogenic epidermal layer was removed with a Padgett electric dermatome at 0.375 mm. Hemostasis was obtained by spry applications of adrenalin diluted in a saline solution at a concentration of 4 mg/L (2 mg/L in children). The wound bed was again disinfected with wet gauzes soaked in dermis Betadine (1/3 bottle in 1 liter of saline solution) for 10 min; final wash was made with wet gauzes soaked in saline solution. Fibrin-cultured autografts (cells on the top) were then carefully placed on the remaining allogermis with the aid of two forceps. Grafts were protected with two layers of Vaseline gauzes (Adaptic; Johnson & Johnson Medical Inc., Arlington, TX), followed by several layers of dry gauzes. Betadine cream was applied around the grafted areas to create a barrier to the external contamination. A usual bandage was used for dressing immobilization. Dressings were made under surgical management: dry gauzes were removed, and grafts covered by Vaseline gauzes were inspected. Swabs were randomly taken for evaluation of bacterial contamination, to have a meticulous monitoring of the systemic antibiotic therapy. Betadine cream was used at the periphery of the grafted areas. After 30 min of air exposure, new dry gauzes were applied together with usual bandages.

Take down was evaluated 8-9 days after grafting: secondary dressings were removed, grafted areas were soaked in saline solution, Vaseline gauzes were carefully removed with the aid of forceps, and take was evaluated after at least 15 min of air exposure. The grafted areas were then covered with nonadherent wound dressing (Silicone N/A; Johnson & Johnson Medical Limited, Gargrave, Skipton, UK). Further dressings (including the barrier to bacterial contamination) were performed as described above. The final evaluation of epidermal regeneration was made 1 month after grafting.

Transmission electron microscopy. Skin specimens were processed for transmission electron microscopy according to standard techniques (13). Semithin and ultrathin sections were cut on an ultramicrotome (Reichert Ultracut E, Leica, Wien, Austria). Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a transmission electron microscope (CM100; Philips, Eindhoven, The Netherlands).

RESULTS

Formation of the fibrin substrate. Fibrin sealant is a plasma-derived product obtained by the mixing of fibrinogen and thrombin (see 46 for a recent review). In preliminary experiments, we noticed that the mixing of fibrinogen and thrombin performed following the manufacturer’s instructions induced a rapid polymerization of fibrinogen, impeding the formation of a transparent fibrin film suitable for cell culture. The rate of fibrinogen polymerization is regulated by the concentration of thrombin (46), whereas the elasticity and transparency of the fibrin polymer is strictly dependent on the ionic strength of the solubilization buffer (Ronfard and Barrandon, Ecole Normale Superieure, Paris, France, personal communication, 1995). The lowering of thrombin from 250 to 1.5 IU/ml, the addition of NaCl at a final concentration of 1.1%, and the lowering of CaCl² from 20 mM to 1 mM allowed the formation of a transparent and elastic fibrin film suitable for keratinocyte cultivation (Fig. 1A).

Evaluation of the keratinocyte clonogenic and growth potential. Hitherto, keratinocyte clonal growth and/or preservation of epidermal holoclonies has been unambiguously demonstrated only when keratinocytes were cultivated on plastic, following the original Rheinwald and Green technique (6, 9-13, 17). We therefore evaluated the clonogenic and growth behavior of fibrin-cultured keratinocytes, using the standard Rheinwald and Green technique as the reference method. Keratinocytes were isolated from skin biopsy specimens of healthy volunteers and cultivated as described (12, 17, 18) (see Materials and Methods). Subconfluent primary cultures were then passaged on either fibrin or plastic, in the presence of lethally irradiated 3T3-J2 cells. Figure 1 shows keratinocyte colonies growing on plastic (panel C) or on fibrin (panel D), 6 days after plating. The overall size and morphology of colonies were similar, the only difference being that cells cultured on fibrin were more packed and showed less defined cell boundaries (compare panels C and D). To determine the growth rate of keratinocytes, subconfluent primary cultures were plated at a density of 6.5×10³ cells/cm² and counted at different time intervals. As shown in Figure 1B, the growth rates of keratinocytes grown on plastic (open circles) or on fibrin (closed circles) were identical. Three different cell strains...
were examined with similar results, and the average cell population doubling time was of approximately 21 hr in both conditions.

Human keratinocytes plated onto a 3T3 feeder layer form colonies, each colony being the progeny of a single cell (17). This allows the precise evaluation of the basal keratinocyte clonogenic ability (CFE), which can be defined as the percentage of cells able to generate colonies. As shown in Figure 1, the CFEs derived from keratinocytes grown on plastic (panel E) or on fibrin (panel F) were very similar (34.6% vs. 39.7%). The clonogenic ability and the growth potential of basal keratinocytes are, however, two very different concepts. The former indicates the capacity of a basal cell to found a colony, the latter deals with its capacity of producing cell generations, hence it deals with its self-renewal potential (see also below). Aborted colonies, for example, are generated by cells with very limited growth potential (terminal cells) (6), and their relative percentage is inversely related to the overall residual growth potential of a keratinocyte strain. The percentages of aborted colonies (calculated as in refs. 6 and 12 in Figure 1, E and F, were of 3.4% and 4.0%, respectively, suggesting the preservation of keratinocyte growth potential on fibrin.

This was formally proven by the number of cell progeny generated during keratinocyte serial propagation. Keratinocytes cultivated on plastic underwent 153.2 cell doublings before losing their proliferative ability. Keratinocytes cultivated on fibrin generated 154.7 progeny before senescence.

Clonal analysis of epidermal stem cells. The direct demonstration of the preservation of stem cells on fibrin was obtained by clonal analysis of subconfluent secondary keratinocytes (see Materials and Methods). Figure 2 shows two colonies (h) isolated from keratinocytes (strain K49) plated on plastic (panel A) or on fibrin (panel B), both generated by holoclones. Their surface area was of 0.42 mm² and 0.55 mm², corresponding to approximately 750 and 990 cells, respectively (6). A quarter of these colonies generated 173 and 151 large and smooth daughter colonies, respectively (panels C and D). The percentage of aborted colonies (6) was 0.53% and 1.2%, respectively. Serial cultivation was carried out for more than 4 months and both clones produced more than 130 generations before senescence. These data fulfill the criteria for holoclones arising from stem cells. A total of 37 and 39 clones obtained from keratinocytes cultivated on plastic and fibrin, respectively, were analyzed. As shown in Figure 2E, the relative percentage of holoclones, meroclones, and paraclonal was similar in these two culture conditions, suggesting that fibrin did not accelerate clonal conversion.

Taken together, these data demonstrate that fibrin represents a suitable carrier for human keratinocytes destined to autologous transplantation in full-thickness burns. We therefore decided to cover massive full-thickness burns entirely with the composite AD/F-CEA technique. Treatment of massive full-thickness burns with the composite AD/F-CEA technique. Seven patients suffering from burn injuries covering 20% to 75% of their body surface (Table 1) were included in this study. The patients' ages ranged between 20 months and 30 years. Full-thickness burns (20% to 45% of the body surface) were deeply excised down to viable tissue (fat or muscle), and the receiving wound bed was prepared using donor skin (37-39). Full-thickness burns covered with fibrin-cultured autografts showed complete (100%) epidermal regeneration was stable as shown in Figure 3 (F and G).
regeneration at 1 month follow-up, with the exception of patient K84 (Table 1), who required a split-thickness meshgraft to cover the remaining 20% of the burned area (Table 1). Therefore, five of seven patients did not require additional surgery or graft application on lesions treated with fibrin-cultured autografts.

In all patients, regenerated epidermis was stable after 2-20 months of follow-up (Table 1). Taken together, these data demonstrate that cultured autografts bearing stem cells can indeed rapidly and permanently cover a large body surface.

Histology and electron microscopy. The neoeipidermis and the dermo-epidermal junction were examined from punch biopsy specimens taken 4, 8, and 12 months after grafting from patients K68, K60, and K50, respectively. In all specimens studied, the epidermis appeared fully differentiated with a well-developed stratum granulosum (Fig. 4, A-C). Compact to basket-weaved hyperkeratosis of the stratum corneum was present at 4 and 8 months after grafting. Neither dyskeratosis nor parakeratosis were observed at any time point. The dermo-epidermal junction appeared almost completely flattened at 4 months, while shallow but clearly defined and evenly distributed rete ridges were evident at 8 months after grafting and became of almost normal depth by 12 months (Fig. 4, A-C).

On electron microscopy examination, all the structural components of the dermo-epidermal junction, i.e., the hemidesmosomes-anchoring filament complexes, the basal lamina, and the anchoring fibrils, were detected at each time point (Fig. 5, C-E). At 4 months after grafting, hemidesmosomes regularly showed a normal tripartite structure with subbasal dense plates and cytoplasmic outer and inner plaques with tonofilament insertion, while the basal lamina appeared continuous but of irregular thickness; anchoring fibrils, although clearly recognizable by their cross-banded pattern, were significantly thinner and sparser than in normal skin (Fig. 5C). After 12 months, however, anchoring fibril structure, size, and density were almost comparable to those of normal control skin (Fig. 5E).

In all specimens, melanocytes with well-developed dendritic processes appeared evenly distributed within the basal epidermal cells, often hanging down into the papillary dermis (Fig. 5A). The presence of numerous mature melanosomes within the basal and, to a lesser extent, the first suprabasal cell layer indicated the reconstitution of the epidermal melanin unit already at 4 months after grafting.

Langerhans cells, the bone marrow-derived antigen-presenting cells of stratified epithelia that are known to be lost during keratinocyte cultures, were regularly detected in the epidermis at each time point and appeared particularly rich in Birbeck granules and cytoplasmic organelles (Golgi complex, rough endoplasmic reticulum, endosomes) (Fig. 5B). At 4 months after grafting, these cells were often located in the first suprabasal cell layer or even within basal keratinocytes, suggesting recent migration from the underlying wound bed.

Below the grafted epidermis, the presence of numerous fibroblasts with dilated cisternae of rough endoplasmic reticulum, capillaries, and interwoven collagen bundles indicated tissue remodeling. The persistence of the allodermis at 4 and 8 months was shown by the finding of residual elastic fibers both in semithin sections and at the ultrastructural level. However, at 12 months after grafting, almost no elastic fibers could be detected, suggesting complete remodeling of the donor dermis; collagen showed a bilayered distribution with fine fibers in the subepidermal region and thicker fibers in the deep dermis.

Long distance transportation, timing, and production cost. Confluent sheets of fibrin-cultured keratinocytes were stirred at room temperature for 12-72 hr, then processed for CFE assays. Keratinocytes cultured on fibrin maintained the clonogenic potential unaltered for at least 72 hr. Instead, cultured epidermal sheets, detached with dispase and mounted on a Vaseline gauze, showed a decrease (of approximately 15%) of the colony-forming ability after 24 hr, with substantial loss (of more than 60%) of the colony-forming ability after 36 hr. These data demonstrate that the clonogenic basal layer, including the progenitor cells, are protected by the continuous adhesion to the fibrin substrate during transportation and handling.

The use of the original Rheinwald and Green technique requires the epithelium to be confluent before detachment (18), whereas the use of the fibrin substrate allows more flexibility in the timing of graft application, because subconfluent cultures can also be applied. This allows shortening of the time between biopsy and graft preparation (see also Table 1) and allows an easier coordination between cell cultivation and surgery.

Cultivation of keratinocytes on the fibrin substrate has profound influences also on the production cost of the cultured autografts, because (i) it avoids the time-consuming procedure for the detachment of the cultures; (ii) it requires less technical support for the preparation of the grafts; and (iii) it avoids the dispase-dependent shrinking of the epidermal sheet. Indeed, the fibrin substrate allows the production of 144-cm² grafts using the same number of clonogenic cells and the same amount of tissue culture media previously needed to produce 50-75 cm² grafts (31, see Discussion). Based on the production cost of our cultures, we have calculated a mean patient cost of approximately US $600 for each 1% body surface covered with fibrin-cultured autografts and of approximately US $1,350 for each 1% body surface covered with plastic-cultured autografts.

Taken together, these data demonstrate that fibrin substantially improves the problems related to the preparation, cost, and transportation of cultured autografts.
More than 5000 people die each year in the United States from massive full-thickness burns (35). Once initial burn shock, hypermetabolic response, and infection are adequately controlled (35, 47), the quick coverage of the skin defects becomes compelling. Autologous split-thickness mesh graft is currently (and rightly) considered the ideal skin replacement (36). However, when donor sites are not sufficient to prepare enough mesh grafts, the permanent coverage of burn wounds with cultured autografts becomes life saving.

The importance of epidermal stem cells. Despite several reports questioning the real usefulness of cultured keratinocytes in massive full-thickness burns (for reviews, see 25, 28, 35, 36 and references therein), data presented in this article demonstrate that cultured autografts bearing holoclones can indeed rapidly and permanently cover a large body surface. Six of seven patients showed a 100% final take of cultured keratinocytes. One might argue that because epidermal stem cells have been pulled out from their natural "niche" (3) and forced to undergo rapid proliferation, they have irreversibly lost their "stem-ness," hence holoclones should not be considered as representative of the in vivo stem cell compartment. However, permanent epidermal regeneration obtained with cultured keratinocytes in massive full-thickness burns (20, 30, 31 and Table 1) formally proves that epidermal stem cells can indeed be preserved in culture and can maintain their stemness also after transplantation. Thus, because holoclones are endowed with the highest proliferative potential in vitro (6, 11-13) and account for the entire proliferative capacity of the original mass culture destined to transplantation (6, 11-13), we feel quite confident in considering them as bona fide keratinocyte stem cells. The concept of holoclones arising from stem cells is further confirmed by recent data obtained from the analysis of the epithelium covering the ocular surface. In vivo data have clearly shown that in corneal epithelium, stem cells are segregated in the limbus, whereas central and paracentral cornea are covered with transient amplifying cells endowed with a different capacity for multiplication (8, 48). Accordingly, corneal holoclones are strictly segregated in the limbus (49), and their cultivation is essential for stable regeneration of corneal epithelium by cultured corneal autografts (7).

This said, during the last few years, new culture technologies have been proposed (for review see 15, 44, 50), envisaging new culture media and/or cultivation of epidermal cells onto different carriers, such as fibrin glue (45), collagen-based "skin equivalents" bearing fibroblasts (51), or hyaluronic acid derivatives (52), even with cells in suspension (53). Maintenance of holoclones has never been demonstrated in these conditions. It should be considered, however, that irreversible holoclone to meroclone to paraclone conversion occurs slowly during natural aging in vivo (2, 6) as well as during serial keratinocyte cultivation in vitro (6, 12, 13). Incorrect culture conditions can irreversibly accelerate the clonal conversion, hence can cause a rapid disappearance of stem cells (our unpublished data), rendering the cultured autograft transplantation useless.

Therefore, the proposal of a new culture system and/or of a new carrier (as for fibrin) for autologous keratinocytes destined to the coverage of massive full-thickness burns should be preceded by (i) the direct demonstration of the presence of holoclones in culture, (ii) the periodical clonal analysis of a reference strain of keratinocytes (both in terms of clonogenic and growth potential), and (iii) the evaluation of the percentage of aborted colonies during cultivation.

In our opinion, these basic "quality controls" eliminate one important hitherto uncontrolled variable in the evaluation of cultured autograft clinical performance and should represent a starting point for improving keratinocyte cultivation, to achieve more robust, reliable, and inexpensive epidermal grafts. It is also worth noting that the evaluation of stem cells is probably not essential when cultured keratinocytes are used as biological medication for inducing re-epithelialization of chronic leg ulcers or partial-thickness burns, because in this clinical context permanent take is not required (31, 54).

The fibrin "delivery system.". The fibrin sealant is degraded very rapidly after grafting (data not shown) and is therefore suitable as a keratinocyte "delivery system." As shown above (see Results), the fibrin sealant allows a better coordination between culture timing and surgery, an easier handling of the keratinocyte cultures, an easier long distance transportation, and, more importantly, a significant decrease of the cultured autograft cost. Based on a selling price of US $15/cm², Rue et al. (42) reported a mean patient cost of US $13,000 for each 1% body surface covered with cultured autografts. This is an exorbitant cost, but it could also be due to the poor clinical results reported in that study. Data presented by Munster (27) reported triplication of the cost in the cultured autograft-treated group of patients as compared with the group of patients treated conventionally. However, in the cultured autograft-treated patients, mortality was reduced by a factor of three (27), suggesting that the average daily cost was actually comparable in the two group of patients. Meuli and Raghunath (29) reported a cost of US $1,650 for each 1% body surface covered with cultured autografts, a value almost 10 times lower than that reported by Rue et al. (42). We do not sell cultured autografts, hence we cannot report a selling price. Based on the production cost of our cultures, however, we have calculated a mean patient cost of approximately US $600 for each 1% body surface covered with fibrin-cultured autografts.

Allogeneic dermis. The importance of allogeneic dermis in determining the take of cultured autografts has been previously highlighted (see 27, 36-39, 41) and is confirmed by our data. Donor skin requires approximately 14 days...
for stable engraftment and safe removal of the donor epidermal layer. Currently, the optimization of keratinocyte cultivation allows a time interval of 14-16 days between skin biopsy and cultured autograft transplantation (Table 1). Therefore, when the composite AD/F-CEA technique is used, it is basically pointless to further shorten the keratinocyte cultivation time. Histology and electron microscopy indicated that, when keratinocytes are applied onto allogeneic dermis, both rete ridge development and regeneration of anchoring fibrils (see Figs. 4 and 5) are accelerated when compared with wound beds devoid of homograft dermis (20, 41), conferring a faster stability to the epithelium. Donor dermis decreases retraction problems and scarring usually observed when keratinocytes are applied directly on the muscular fascia or on granulating tissue (31). Electron microscopy data suggest that allogermis is completely remodeled by the host in approximately 1 year.

It is worth stressing that carriers such as collagen or fibrin or hyaluronic acid should be considered just as keratinocyte "delivery systems," even if they are inoculated with fibroblasts. Often, these carriers are misleadingly advertised as "dermal substitutes," inspiring the belief that they could actually substitute for the missing dermis in a full-thickness burn. Dermis is a very complex structure, divided in at least two parts (the pars papillaris and the pars reticularis) containing at least two types of fibroblasts with distinct specialized functions, different types of collagen fibrils, elastic fibers and proteoglycans, and harboring skin appendages (and relative specialized cells), blood vessels, and nerves. Recently, several "dermal equivalents" such as collagen sponge skin equivalent (55) or synthetic mesh skin equivalent (56) have been proposed as alternatives to cryopreserved cadaver skin for the preparation of the wound bed. Randomized withinpatient controlled clinical trials with a long-term clinical and histological follow-up must be performed in numerous burn units and by independent investigators to assess their true clinical efficacy.

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* Abbreviations: AD/F-CEA, allodermis/fibrin-cultured autografts; CFE, efficiency of colony formation; DMEM, Dulbecco-Vogt Eagle's medium. [Context Link]