Burn Wound Closure With Cultured Autologous Keratinocytes and Fibroblasts Attached to a Collagen-Glycosaminoglycan Substrate

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Improved survival of patients with extensive burn injuries requires the development of better techniques for timely and permanent closure of the full-thickness wound. We developed procedures for establishing confluent, stratified layers of cultured, autologous keratinocytes on the surface of a modified collagen-glycosaminoglycan membrane that contains autologous fibroblasts. We transferred the composite grafts onto limited areas of excised full-thickness burn wounds of four patients with extensive burn injuries. Our results indicate that this composite graft material has acceptable "take," with formation of a basement membrane within 9 days of graft placement. Use of this technique for major wound coverage may offer a significant advance in the care of extensively burned patients and also may be useful for providing skin for reconstructive surgery.

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EARLY wound excision and prompt wound coverage in severely burned patients shortens hospital stay and ultimately may be shown to lessen morbidity and improve survival.[1,2] Although the widespread use of allograft skin allows for temporary wound coverage, the achievement of timely, permanent coverage of the excised wound remains a problem in extensively burned patients who have limited donor sites for autograft harvesting. Limited success has occurred with the use of immunosuppression to allow extended allograft survival,[3-5] but infectious complications have limited widespread adoption of this technique. More recent experiences with artificial skin, composed of a collagen matrix–Silastic membrane,[6,7] or with cultured epidermis[8-13] have achieved wound closure in some patients. However, use of a collagen-Silastic composite material must be followed by conventional autografting to achieve permanent wound closure. While cultured epidermal sheets have been successful in some cases, the general experience has been that keratinocyte sheets do not attach and vascularize sufficiently well on wound beds to achieve reproducible and reliable wound closure.[11] In addition, the resultant flat epidermal-dermal junction is slow to develop rete ridges, and epithelium may detach easily from the wound bed subsequent to grafting.[9-13]

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We developed a process for achieving the attachment of cultured human keratinocytes (HKs) to the surface of a cross-linked, collagen-based substrate. Confluent, membrane-bound HKs then can be transferred to the wound surface, where the collagen matrix can serve as a framework to allow fibrovascular ingrowth from the underlying wound bed to permit HK survival and the formation of a neodermal structure.[14,15] We demonstrated that successful attachment of cultured HK to collagen matrices can be achieved in vitro,[16] and vascularization of these composite grafts employing HK can be achieved in wounds on athymic mice.[17,18] We report clinical trials with this material in four patients, using autologous HKs and human fibroblasts (HFs) for preparation of the composite grafts.

MATERIALS AND METHODS

Preparation of Composite Grafts

Composite grafts were made from separate, parallel cultures of autologous HKs and HFs and acellular collagen-glycosaminoglycan (C-GAG) membranes. Dermal membranes were constructed similar to methods of Yannas et al,[19] with modifications. The structure of the C-GAG membrane was optimized for pore size to promote ingrowth of fibrovascular tissue from the wound bed, while keeping the HKs on the

membrane surface.[15] A nonporous surface of C-GAG was laminated to the membranes to provide a planar surface for cultured HKs.

A sample of partial-thickness skin tissue (approximately 5×5 cm) was obtained from the burned patient as early as possible after hospital admission. Proliferative HFs and HKs were isolated by enzymatic digestion of this tissue and expanded in number in polystyrene tissue culture flasks as described.[20-22] Human keratinocytes were cultured in serum-free MCDB 153 with increased concentrations of six amino acids and HFs were grown in Dulbecco's modified Eagle's medium supplemented with 10% (percent volume in volume) fetal bovine serum plus epidermal growth factor (Gibco Laboratories, Grand Island, NY) (10 μg/L), insulin (5 mg/L), and hydrocortisone (0.5 mg/L). Subconfluent primary cultures were ready for harvest after 9 to 12 days and were subcultured onto a surface area of 1.50×10^3 to 2.25×10^3 cm^2. Secondary cultures were ready for inoculation onto C-GAG membranes within 1 week of subculture.

The C-GAG membranes (10×10 cm) were removed from storage in 70% isopropyl alcohol,[15] transferred into 150-cm diameter Petri dishes, washed repeatedly in *N*-2-hydroxyethylpiperazine-*N*-2 -ethanesulfonic–buffered saline solution, and preincubated in the solution overnight at room temperature to elute any remaining isopropyl alcohol. The saline solution was replaced with three changes (30 minutes each) of supplemented Dulbecco's modified Eagle's medium, and membranes were equilibrated in Dulbecco's modified Eagle's medium in an incubator (37°C, 5% carbon dioxide, saturated humidity) for 30 to 60 minutes. Subconfluent cultures of HFs were inoculated at a density of 3.7×10^5 to 6.9×10^5 cells/cm^2 into the porous (internal) surface of the C-GAG membrane. Medium was changed daily. After 1 to 3 days, supplemented Dulbecco's modified Eagle's medium was replaced with serum-free MCDB 153 that contained 0.2 mmol/L of calcium.[22] Subconfluent HKs then were inoculated at a density of 0.54×10^6 to 1.0×10^6 cells/cm^2 onto the nonporous, laminated surface of the C-GAG membrane and incubated for a minimum of 4 additional days. For the last 48 hours of incubation, the calcium concentration in the medium was elevated to 0.3 mmol/L. For the final 24 hours of incubation, epidermal growth factor and bovine pituitary extract in the medium were replaced with recombinant human basic fibroblast growth factor (Amgen Biologicals, Thousand Oaks, Calif) at a concentration of 100 μg/L. For clinical application, excess culture medium was removed from the culture dish and a sheet of N-Terface (Winfield Laboratories, Richardson, Tex) was placed over the graft to facilitate the transporting and securing (suturing or stapling) of the graft to the wound bed. (N-Terface is a polypropylene, nonadhering mesh material that detaches easily from the graft at the first dressing change.)

Application of Composite Grafts to Wounds

After fluid resuscitation was completed, patients underwent early burn wound excision to subcutaneous fat or fascia. This required from one to three separate procedures, depending on the total burn size, and usually was carried out on successive days. Excised wounds were covered immediately with autograft or allograft (fresh or cryopreserved) skin to achieve wound closure. Subsequently, patients were returned to surgery as fresh donor sites became available and patient condition permitted. Allograft skin was removed from areas to be autografted, hemostasis of the wound bed was achieved using standard techniques, and a small (1 to 2 g) portion of wound was excised for microbiological culturing. Composite grafts, with the epidermal side adjacent to N-Terface, were transferred to selected wound areas and the N-Terface was stapled or sutured in place over the skin substitute. Grafts then were covered with a petrolatum gauze (Xeroform, Sherwood Medical, St Louis, Mo) and bulky gauze dressings. Grafts were inspected 5 to 6 days after surgery and at periodic intervals thereafter.

Histological Preparation and Examination of Biopsy Samples

Biopsy samples were collected for light and electron microscopy at 9 days and 3 to 4 weeks following graft placement. Biopsy sites were greater than 4 cm from the perimeter of the cultured skin graft to minimize the possibility that epithelium had migrated over the wound from the wound perimeter.

Light Microscopy

Tissue samples for light microscopy were fixed in 10% formalin, embedded in paraffin, sectioned at 3- to 6-μm thickness, mounted onto slides, and stained with hematoxylin-eosin. Skin substitutes before placement onto wounds also were examined histologically.

Electron Microscopy

Samples for electron microscopy were fixed in Trump's fixative and in 1% osmium tetroxide, dehydrated, infiltrated and embedded in epoxy resin, thin sectioned, mounted onto grids, and stained with lead citrate and uranyl acetate. Microscopy was performed on a transmission electron microscope (Hitachi, Nissei Sangyo America, Mountain View, Calif) operated at 75-kV accelerating voltage.

RESULTS

Patient Information

Patients agreed to participate in the study after informed consent was obtained to a protocol approved by the University of California at San Diego institutional review board. Information regarding the four patients treated is summarized in the Table. The body surface area of burn (partial- and full-thickness injury) ranged from 43% to 74%. Cultures of HKs and HFs were initiated from 1 to 4 days after burn injury. Two to five cultured grafts (8.5×8.5 cm) were applied to each patient.

Histology

The typical histological appearance of the skin substitute used in this study is shown in Fig 1, top, immediately before application to the wound. The reticulated C-GAG matrix defines the thickness and porosity of the skin substitute. This composite material differs from earlier examples[16-18] by the addition of HFs to the reticulated interior of the C-GAG membrane. After application, epithelial foci are evident at the time of the first dressing change after surgery (5 to 6 days). By the second dressing change (9 days after surgery), epithelial areas are well keratinized and beginning to desquamate. Histological examination of epithelialized areas on day 9 (Fig 1, center) confirms formation of a fully differentiated epithelium and also shows remnants of the C-GAG substrate. The dermal component of the regenerating skin has developed sufficient vascular supply to support the epidermis and has become populated by noninflammatory connective-tissue cells, predominantly fibroblasts. Figure 1, bottom, shows restoration of relatively normal histiotypic anatomy of skin healed after 4 weeks.

Unique to the study is the early formation of a well-interdigitated, undulating dermal-epidermal junction together with a flat, relatively smooth epidermal surface. The interdigitated epidermis provides a greater dermal-epidermal junction per unit of external surface area than occurs with the formation of a flat epidermis as described with epidermal sheet grafts.[8,11,23-25] The increased area of the junction may contribute to the strength of the healed

Patient Information

	Patient No.*				
	1	2	3a	3b	4
Body surface area burned, %	43	40	74	74	49
Sex	F	M	M	M	M
Age, y	53	22	20	20	39
Postburn day primary culture initiated	4	2	2	2	1
Postburn day cultured graft placed	23	23	21	30	20
No. of grafts	2	3	3	2	3
No. of grafts surviving	1	2	2	2	2
Location	Chest (×2)	Right leg, fail; Left abdomen, survive	Left abdomen (×2), survive; Right abdomen, fail	Left thigh (×2), survive	Left arm (×2), survive; Left shoulder, fail
Operative culture†	HG	Right leg, HG; Left abdomen, MG	Left abdomen, NG; Right abdomen, HG	HG	NG
No. of cultured human fibroblasts ($\times 10^5$) inoculated/cm²	3.70	5.88	5.88	6.92	5.77
No. of cultured human keratinocytes ($\times 10^5$) inoculated/cm²	6.02	7.34	5.40	8.17	10.1

*Patient 3 had two separate applications of grafts.
†NG indicates no growth in wound bed at time of graft application; MG, moderate growth (<100000 organisms per gram of tissue); and HG, heavy growth (>100000 organisms per gram of tissue).

epithelium.[26] This result was observed in biopsy samples from all four patients.

Physical Characteristics of Healed Wounds

Gross examination of a treated area at 4 weeks after surgery shows a soft, relatively smooth external surface that has good subjective resistance to shear forces (Fig 2). Focal redevelopment of pigment was seen in one (black) patient at 4 weeks after surgery. We observed similar results with skin substitutes on athymic mice.[18] Although small areas of focal ulceration appear during the first 3 weeks of epithelialization, the extensive epidermal sloughing that is reported to occur after grafting with epidermal sheets alone[9-13] did not occur in healed wounds.

Ultrastructure of the Dermal- Epidermal Junction

Examination by transmission electron microscopy of the dermal-epidermal junction at 9 days after surgery shows development of a continuous lamina densa and multiple hemidesmosomes (Fig 3, top). This result is distinguished from reports by Woodley et al[11] with epidermal-only sheet grafts and by Langdon et al[27] with dermal-epidermal composite grafts that were applied in two stages. Early formation of basement membrane ultrastructure after grafting is consistent with reports from our laboratory of biologic attachment of cultured HK to C-GAG substrates in vitro.[16] Initiation of basement membrane formation in vitro and elimination of the requirement to release epidermal sheets from tissue culture flasks by enzymatic digestion immediately before grafting may account for early restoration of these histological characteristics of the junction.

Also unique to this report is early formation of anchoring fibrils between healed epithelium and regenerated connective tissue (Fig 3, bottom). Formation of anchoring fibrils requires biologic components from both keratinocytes and fibroblasts. Inoculation of C-GAG membranes with fibroblasts and keratinocytes in vitro may contribute to early postsurgical establishment of this structure that is critical for epidermal adherence.

Graft Failures

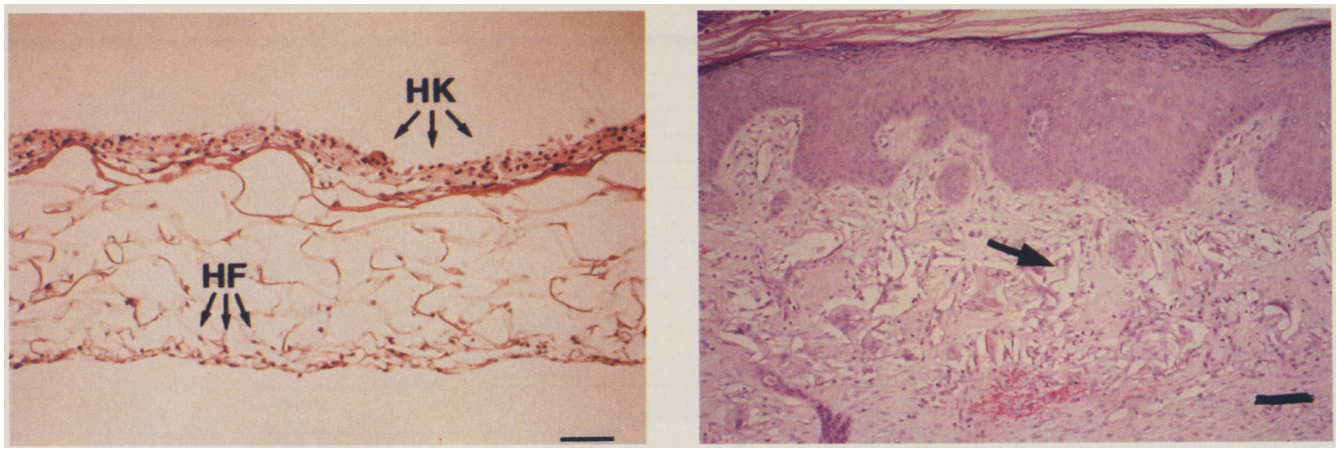
Four of 13 grafts that were placed failed to take. By 5 to 7 days after graft placement in these cases, the composite graft was covered with exudate and appeared to have "dissolved." As noted in the Table, graft failure seemed to correlate with colonization of the underlying wound bed with moderate to heavy growth of microorganisms. Colonization of the underlying wounds was surprising to us since in all cases allograft was well vascularized to the excised wound bed.

COMMENT

Permanent and timely skin replacement after major burn injury remains a significant problem. Although the results of strictly controlled comparison studies are not available in patients with extensive burn injuries, rapid wound closure shortens hospitalization time and ultimately may be shown to correlate positively with patient survival.[28,29] The widespread use of human cadaver skin has allowed for complete albeit temporary wound coverage with a biologic dressing immediately following burn wound excision. However, even with wide-expansion skin-meshing techniques, multiple operative procedures and repeated autograft harvests often are required before complete permanent wound closure is obtained. This may require weeks or months in patients with massive burn injuries. Skin donor sites can contribute their own morbidity (pain, prolonged healing that delays reharvesting, and scarring).

The achievement of improved culture techniques for HKs with rapid cell expansion in vitro has led several groups to attempt to cover full-thickness, excised wounds with cultured cell sheets. Although some success has been achieved,[8,9,12] the acceptance of HK cultured sheet grafts on wounds may be limited,[13] and integrity of the resulting skin structure is less than ideal after grafting, with easy deepithelialization and blister formation reported as late as 7 months after grafting.[9-11,23]

Previous reports indicate that cultured HKs might lose antigenicity or HLA-DR expression to the extent that long-term or permanent acceptance of allogeneic HK grafts might be achieved.[30-32] However, experiments from our collaborative laboratory group have shown that while HLA-DR expression is decreased in cultured HKs, expression can be restored by certain conditions, including exposure to interferon gamma.[33] Our previous use at the University of California, San Diego Medical Center of composite grafts prepared with allogeneic HKs (five patients [J.F.H. and S.T.B., unpublished data, 1988]) produced poor results on full-thickness wounds, similar to the experience of Madden et al.[34] Although successful take of allogeneic HK sheets on partial-thickness wounds has been reported,[34,35] convincing proof of allogeneic cell persistence is difficult when wounds are capable of autoepithelialization. Allogeneic cells placed on partial-thickness wounds may function as a



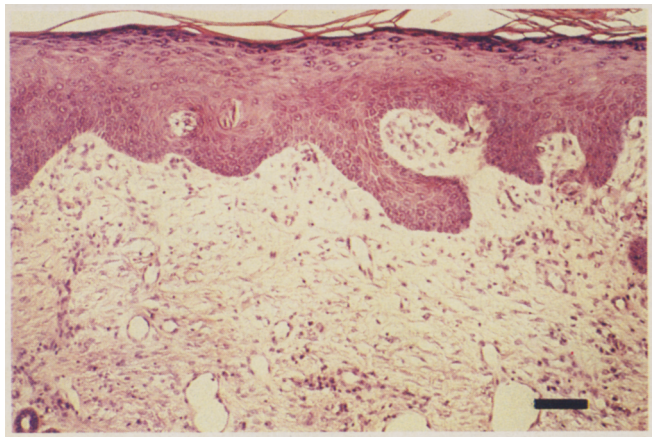


Fig 1.—Top left, Histology of cultured skin substitutes before grafting. Skin substitute consists of autologous, cultured human keratinocytes (HK), seen on the top surface, and fibroblasts (HF), attached to a collagen-glycosaminoglycan (C-GAG) substrate, which appears as a meshwork beneath the HK. Human keratinocytes are restricted to the external surface of the C-GAG matrix, and HFs populate the internal structures of the reticulated pores. Scale bar indicates 0.1 mm (hematoxylin-eosin, ×25). Top right, 9 days after surgery, a fully stratified and desquamating epidermis has formed that is interdigitated with the regenerating connective tissue in the wound bed. Reticulated remnants of the C-GAG substrate are seen among the regenerating tissue (arrow). Scale bar indicates 0.1 mm (hematoxylin-eosin, ×25). Bottom, By postsurgical day 26, dermal and epidermal components have been restored. Interdigitation of the dermal-epidermal junction resembles rete ridges of normal skin. Pigmentation is present, but not complete, and epidermal adnexi do not form. Scale bar indicates 0.1 mm (hematoxylin-eosin, ×25).

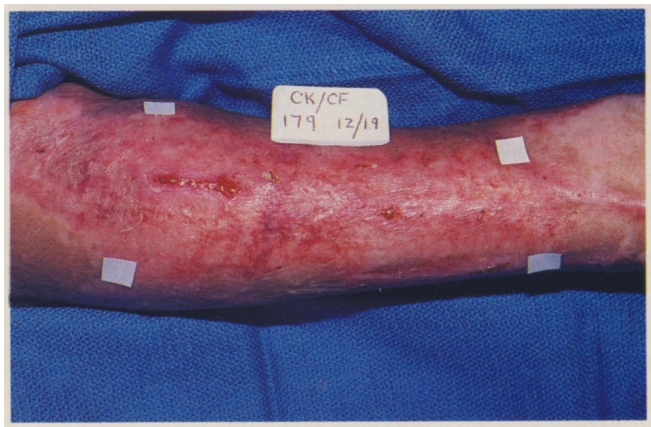


Fig 2.—Appearance of a wound (left forearm) 16 days after closure with cultured skin substitute (area between white markers). External surface of regenerated skin generally is smooth and flat, with some focal ulcerations. The regenerated skin has good subjective resistance to shear. Within 10 days, the ulcerations had closed and the skin surface was smoother.

temporary dressing, but permanent closure of these wounds probably occurs from recipient adnexal structures that ultimately replace the transplanted allogeneic epithelium.

We believe that use of a collagen matrix in these studies has several advantages over the use of epidermal cell sheets alone for wound coverage. Previous work by Bell et al[36] involved the use of keratinocytes cultured on the surface of a collagen gel, with successful wound coverage reported in animals. However, reports of clinical studies with this material are not available. We suspect that the open-matrix framework of our collagen material will allow for more rapid ingrowth of fibrovascular tissue than would be possible with use of a collagen gel.

As an alternative to the use of a collagen matrix or gel as a template for dermal reconstitution, several groups have employed dermal allografts that are then resurfaced with autologous HKs.[37-40] This technique relies on low immunoreactivity of dermal tissue, including fibroblasts and endothelial cells.[41] Use of a shelf-storable, sterilizable dermal matrix offers the advantages of avoidance of disease transmission, consistency of composition, and ready availability. In addition, we believe that establishing adherence of cultured HKs to the collagen membrane in vitro, prior to placement on the wound, offers an advantage over techniques that employ application of HK cell suspensions or cultured HK sheets directly

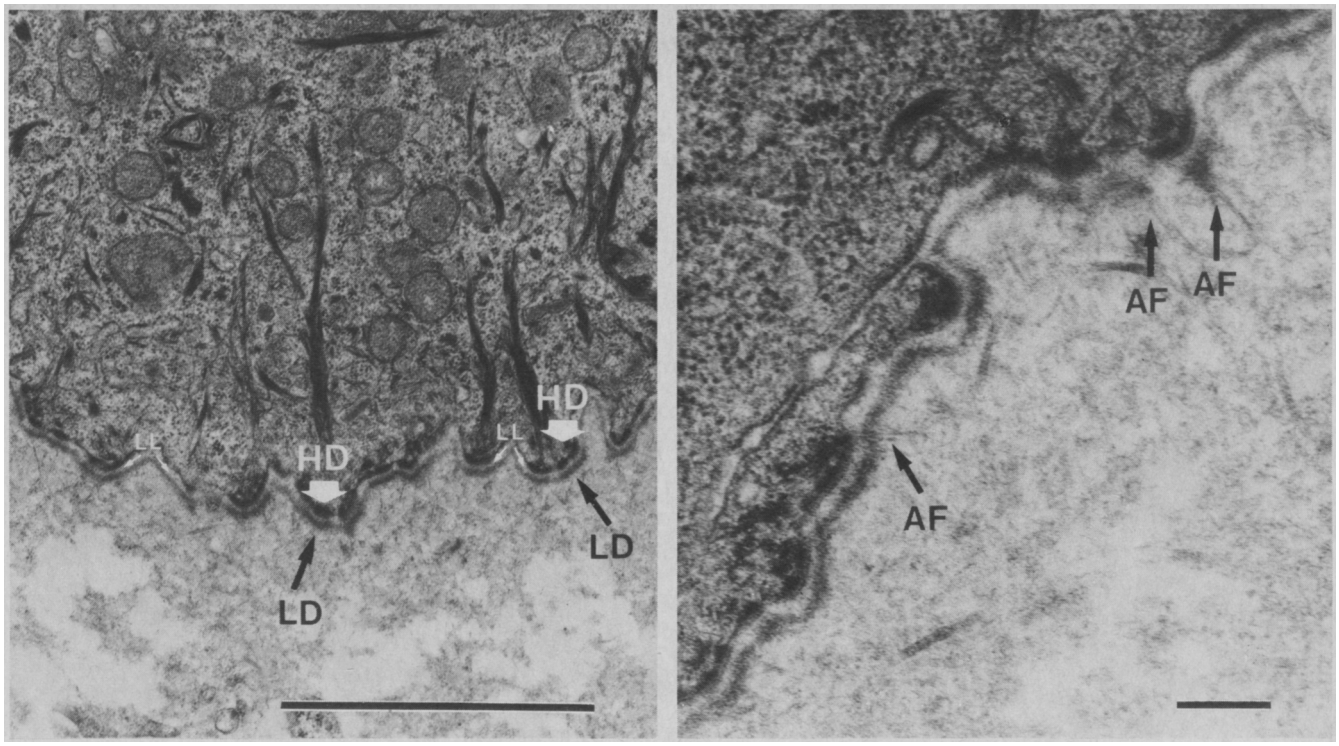


Fig 3.—Transmission electron micrographs 9 days after surgery. Left, Dermal-epidermal junction of skin is healed with cultured skin substitute. Continuous lamina densa (LD) and lamina lucida (LL) are seen at the dermal-epidermal junction. Hemidesmosomes (HD) are seen frequently along the junction. Scale bar indicates 10 μm (original magnification ×3500). Right, Anchoring fibrils (AF) have developed and should strengthen the permanent attachment of the healed epidermis to the regenerated connective tissue. Scale bar indicates 1 μm (original magnification × 1400).

onto the wound. This approach allows application of the composite material in a single procedure rather than in two procedures as required by other techniques.[6,37]

Basement membrane, including formation of lamina densa and anchoring fibrils, is the extracellular material by which epidermis adheres to native dermis or to regenerated connective tissue.[26] Data from other investigators who employed the grafting of autologous HK sheets alone, without a dermal structure, indicate that basement membrane and hemidesmosome formation occur by 3 weeks[24] to 4 weeks[9,25] and that rete ridges do not appear until 5 to 12 months[24] or not at all.[9] In our studies, formation of anchoring fibrils, basement membranes, and hemidesmosomes occurred in all four patients at the first biopsy, 9 days after grafting. All grafts had evidence at that time of epidermal-dermal interdigitation, suggestive of rete ridge formation. Furthermore, the resulting skin had excellent structural integrity by 2 to 3 weeks after grafting. We believe that prior attachment of HKs to the collagen matrix during the culture period facilitates early formation of dermal-epidermal attachments after placement of composite grafts on the wound. We have not examined the in vitro grafts for evidence of early basement membrane structures, since our findings of early basement formation after graft placement were unexpected.

Expansion of graftable material for wound closure is a central motive for use of tissue-cultured cells in burn treatment. Logarithmic increase in numbers of cultured skin cells (HKs and HFs) allows vast amounts (>1000 times the original biopsy sample) of skin material to be produced by 3 weeks after culture initiation.[21,22] In the current studies, maximum tissue expansion was not emphasized, and relatively modest expansions of skin (10 to 20 times biopsy size) were performed. However, this expansion of graftable skin represents substantial improvement over conventional meshed skin autograft and can be increased easily to supply the need for skin of patients with extensive burns.

Storage of these skin substitutes is possible by cryopreservation of the cellular components as suspensions of cells in log phase growth and by dry storage of collagen matrices. Storage can allow preparation of these kinds of skin substitutes for sequential applications during the acute hospitalization and for later reconstructive surgery. Other nonacute applications (ie, plastic and dermatologic surgery) also might be served by these materials.

Early vascularization may be critical to graft survival. To stimulate angiogenesis, basic fibroblast growth factor was substituted for epidermal growth factor and bovine pituitary extract in the culture medium. Basic fibroblast growth factor stimulates angiogenesis[42,43] and also is mitogenic for growth of HKs and HFs in culture.[44-46] Addition of basic fibroblast growth factor to the skin substitutes immediately before clinical application may promote two essential components of skin wound closure: vascularization and epithelialization.

Further studies will involve grafting of larger surface-area wounds with this composite graft material and effective and safe provision of appropriate antimicrobial agents to the wound site to decrease graft loss presumably due to infections.[47] We believe that these preliminary results demonstrate improved quality of skin healed with cultured cells combined with a collagen matrix. We suggest that preparation of large quantities of permanent skin replacement material using cultured HKs and HFs attached biologically to a collagen matrix will reduce the time required for permanent, stable wound closure and may offer an important advance in the care of patients with massive burn injuries.

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