Skin Anatomy and Antigen Expression after Burn Wound Closure with Composite Grafts of Cultured Skin Cells and Biopolymers

Steven T. Boyce, Ph.D., David G. Greenhalgh, M.D., Richard J. Kagan, M.D., Terry Housinger, M.D., J. Michael Sorrell, Ph.D., Charles P. Childress, M.A.T., Mary Rieman, R.N., and Glenn D. Warden, M.D.

Cincinnati and Cleveland, Ohio

Closure of large skin wounds (i.e., burns, congenital giant nevus, reconstruction of traumatic injury) with split-thickness skin grafts requires extensive harvesting of autologous skin. Composite grafts consisting of collagen-glycosaminoglycan (GAG) substrates populated with cultured dermal fibroblasts and epidermal keratinocytes were tested in a pilot study on full-thickness burn wounds of three patients as an alternative to split-thickness skin. Light microscopy and transmission electron microscopy showed regeneration of epidermal and dermal tissue by 2 weeks, with degradation of the collagen-GAG implant associated with low numbers of leukocytes, and deposition of new collagen by fibroblasts. Complete basement membrane, including anchoring fibrils and anchoring plaques, is formed by 2 weeks, is mature by 3 months, and accounts for the absence of blistering of healed epidermis. All skin antigens tested (involucrin, filaggrin, laminin, collagens IV and VII, fibronectin, and chondroitin-sulfate) were expressed by 16 days after grafting. This cultured skin analogue provides an experimental alternative to split-thickness skin graft that develops histiotypic markers of skin anatomy and antigen expression after wound closure. (*Plast. Reconstr. Surg.* 91: 632, 1993.)

Closure of full-thickness skin wounds is accomplished safely and effectively by grafting of split thickness autologous skin as sheets or mesh.[1-3] However, inadequate availability of skin autograft in large total body surface area burns has led to development of alternative materials from ex vivo and in vitro sources[4-11] for repair of epidermal and/or dermal skin. These materials are applied in either one or two surgical procedures. Cultured epidermal autografts[4,5,7] are administered over fascia, granulation tissue, or allo dermis, but are known to blister and ulcerate from slow development of dermal-epidermal junction (DEJ) for several months after grafting.[7,11-13] By contrast, skin analogues with mesenchymal and epithelial components that are applied in one procedure are reported not to blister after regeneration of epidermal tissue[9,10] in greater similarity to split-thickness skin autograft.

To provide maximum availability of skin substitutes for permanent closure of full-thickness burns, composite grafts consisting of cultured autologous keratinocytes and fibroblasts attached to collagen-glycosaminoglycan (GAG) substrates can be prepared consistently and stored conveniently in large quantities.[9,14-17] This pilot study describes initial evidence that normal markers of epidermal differentiation (involucrin,[18] filaggrin[19]), DEJ (collagens IV and VII,[20] laminin[21]), and dermis (fibronectin,[22] chondroitin-sulfate[23]) are expressed by 16 days after grafting of this cultured skin analogue onto full-thickness excised burns in pediatric patients. Microscopic analyses of patient biopsies beginning 2 weeks after grafting confirm development of skin anatomy and ultrastructure corresponding to antigenic markers of normal human skin. Together, these qualitative data demonstrate that a cultured cell-biopolymer implant

From the Shriners Burns Institute, the Department of Surgery at the University of Cincinnati, and Case Western Reserve University.
Received for publication June 19, 1991; revised March 25, 1992.

Presented 04 Apr 91 at the 23rd Annual Meeting of the American Burn Association. Supported by the Shriners of North America, grants #15893 and #15837.

can be applied in a single procedure, and can rapidly regenerate epithelial and mesenchymal tissues for skin wound repair. Early formation of DEJ eliminates complications of skin blistering and ulceration associated with application of cultured epidermal autografts after enzymatic release from tissue culture vessels. Increased availability of cultured skin compared to skin autograft for large skin loss injuries may decrease requirements for donor skin for completion of wound closure.

METHODS AND MATERIALS

Patient Information

Three patients admitted to the Shriners Burns Institute in Cincinnati were enrolled by informed consent into a clinical study of cultured skin grafts (CSG) for burn treatment that was approved by the University of Cincinnati Institutional Review Board (IRB). Patient information is summarized in Table I. Two patients were 3 years old and one was 14 years old at the time of grafting. Full-thickness burn size ranged from 65 to 80 percent, and the size of the area grafted with CSG ranges from 120 to 216 cm^2 (two or three grafts, 8.5 × 8.5 cm each).

Graft Preparation and Composition

Biopsies ranging in size from 10 to 20 cm^2 were collected at the first scheduled skin graft procedure after acquisition of informed consent. Epidermal keratinocytes and dermal fibroblasts were isolated by enzymatic and mechanical disaggregation and cultured in selective nutrient media for each cell type as previously described.[9,14–17,24] After propagation of each cell type to very large numbers, dermal fibroblasts were inoculated onto the porous surface of collagen-GAG substrates.[9,16,17] One day later, substrates were inverted to orient the nonporous lamination on top, and epidermal keratinocytes were inoculated to cover substrate surfaces. After inoculation, cultured cell-collagen-GAG composites were cultured from 4 to 14 days in serum-free medium MCDB 153 either submerged or lifted to the air-liquid interface.[21,25,26] For 24 hours before surgery, grafts were incubated in medium in which 1 to 10 ng/ml epidermal growth factor was replaced with 10 ng/ml basic fibroblast growth factor.

TABLE I
Patient Information

	Patient No.		
	1	2	3
Age	14	3	3
Sex	M	F	M
% TBSA	65	64	80
PBD donor skin	6	6	5
PBD CSG applied	34	31	33, 40
PBD CSG biopsy	48, 118	45, 58, 108	56
CSG site	Abdomen	Left flank	Right knee, back
CSG area (cm^2)	144	120	72, 144
CSG % engraftment*	15	65	15
Microscopy	LM, TEM	LM, TEM	IFM

* Engraftment as CSG was estimated at day 10 after grafting and is expressed as "%" of area with dry, keratinized epithelium on the wound. Abbreviations: TBSA, total body surface area; PBD, post-burn day; CSG, cultured skin graft; LM, light microscopy; TEM, transmission electron microscopy; IFM, immunofluorescence microscopy.

Surgical Application, Dressings, and Wound Care

Sites receiving cultured grafts included the abdomen, flank, back, and knee. Wounds were excised to viable tissue (fat or deep dermis) and soaked overnight in wet dressings with 0.5% Sulfamylon (mafenide acetate) solution (administered under separate IRB approval). The following day, wounds were irrigated well with saline, and composite cultured grafts were placed onto wounds with a backing of N-Terface and stapled to the wound bed. Grafts were then covered either with dry dressings consisting of Xeroform gauze, cotton gauze bolster, and spandex, or with wet dressings consisting of fine mesh gauze, cotton gauze, perforated red rubber catheters (0.5 cm diameter), additional cotton gauze, and spandex stapled to surrounding skin. Wet dressings were irrigated with antimicrobial solutions administered through catheters at 2-hour intervals on a protocol of 0.5% Sulfamylon solution followed by two additions of double antibiotic

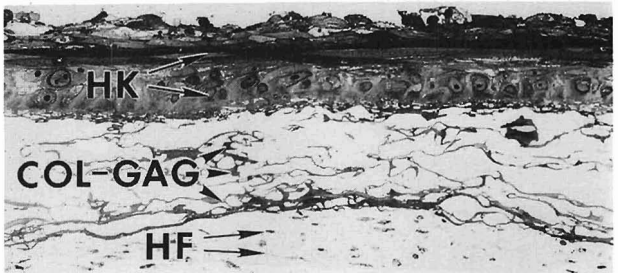

FIG. 1. Histology of cultured skin graft. Composite grafts consist of cultured human epidermal keratinocytes (*HK*) and fibroblasts (*HF*) attached to a thin sponge of collagen-glycosaminoglycan (*COL-GAG*) that has a nonporous film as an epithelial culture surface. The total thickness of the skin substitute is no greater than 0.5 mm (0.020 inches) to minimize time of vascularization. Scale bar = 0.1 mm.

(DAB; 40 μg/ml neomycin sulfate and 200 units/ml polymyxin B sulfate). Wet or dry dressings were changed on day 2 or 3 and on day 5, at which time wet dressings were discontinued. Thereafter, all dressings were changed twice daily with Adaptic and Aquaphor containing bacitracin-zinc ointment (500 μg/gm), or 3 parts bacitracin ointment plus part 1% silver sulfadiazine cream. Grafted wounds were observed at each dressing change until day 5 and once daily until day 10. After reepithelialization was complete, pressure garments were applied to the grafted sites.

Light and Transmission Electron Microscopy (LM and TEM)

Biopsies (3 mm) for LM and TEM were collected under general or local anesthesia from patients after 14 days, approximately 1 month, and approximately 3 months, as indicated in Table I. Biopsies were rinsed in saline, bisected, and fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M Na cacodylate buffer, pH 7.4, and subsequently processed by standard TEM methods.

Immunofluorescence Microscopy and Skin Antigens

Comparison was made of skin tissue regenerated after application of cultured skin grafts to ungrafted cadaver skin (control) by indirect immunofluorescence with a limited panel of monoclonal antibodies to skin antigens. The panel included: (1) involucrin of stratum corneum; (2) filaggrin of stratum granulosum; (3) collagen type IV of basement membrane lamina densa; (4) laminin of lamina lucida; (5) collagen type VII of anchoring fibrils; (6) fibronectin; and (7) chondroitin-sulfate of dermis. All primary antibodies were reacted with unfixed cryostat sections (3–6 μm) of skin samples on glass slides, followed by reaction with biotinylated secondary antibodies,

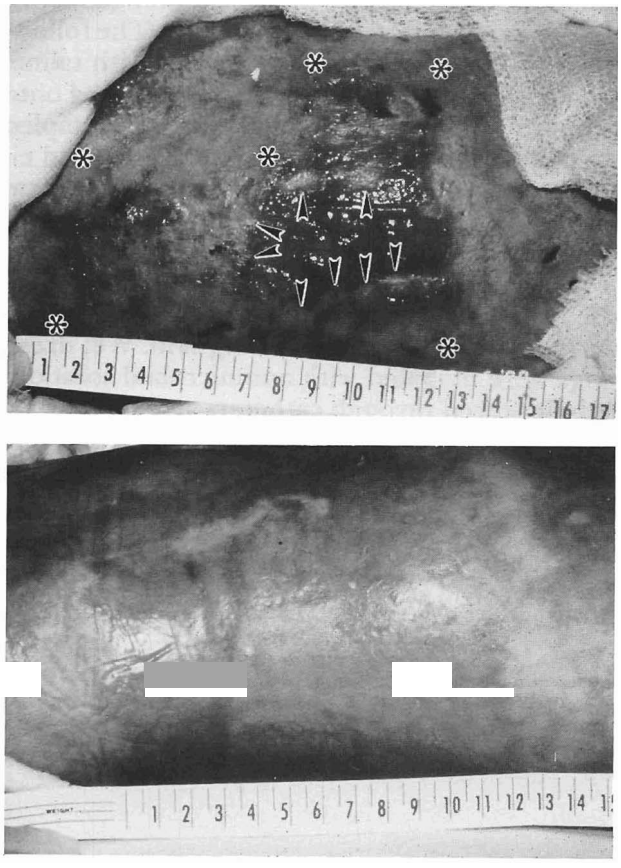

FIG. 2. Clinical result. *Top panel*: Ten days after grafting keratinized epithelium covers greater than 50 percent of the left abdomen (*, treated area), and several islands of keratinized epithelium (*arrowheads*) develop that later grow radially and coalesce to form contiguous epithelium. *Bottom panel*: Six weeks after grafting and use of pressure dressings, the treated area is smooth, remains closed, and does not blister. Scale in centimeters.

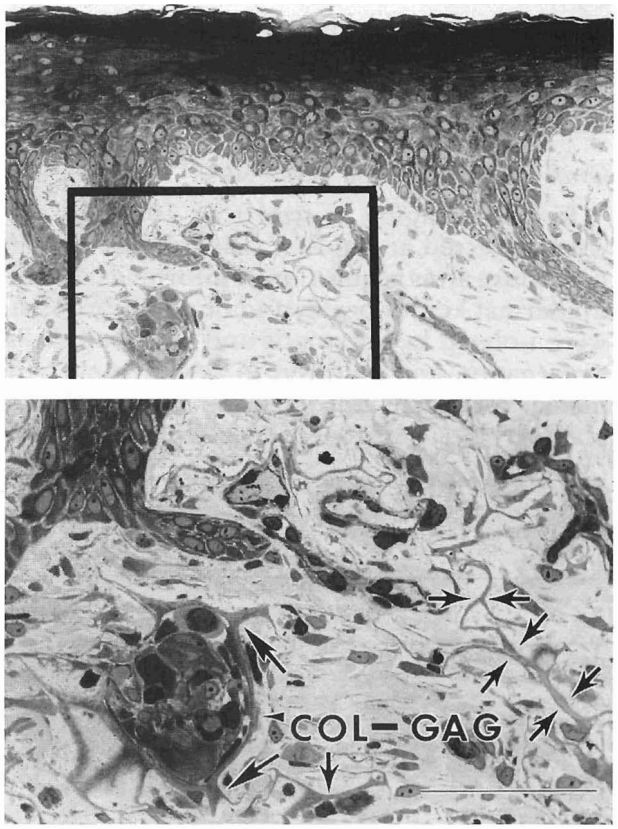

FIG. 3. Histology of healed skin after 2 weeks. Biopsies taken at 2 weeks after application of cultured skin. *Top panel*: Well-keratinized epidermis with initial epidermal hypertrophy and a highly interdigitated dermal-epidermal junction. *Bottom panel*: Identification (*arrows*) of the implanted collagen-GAG substrate with low numbers of associated leukocytes, but not a dense infiltrate. Scale bar = 0.1 mm.

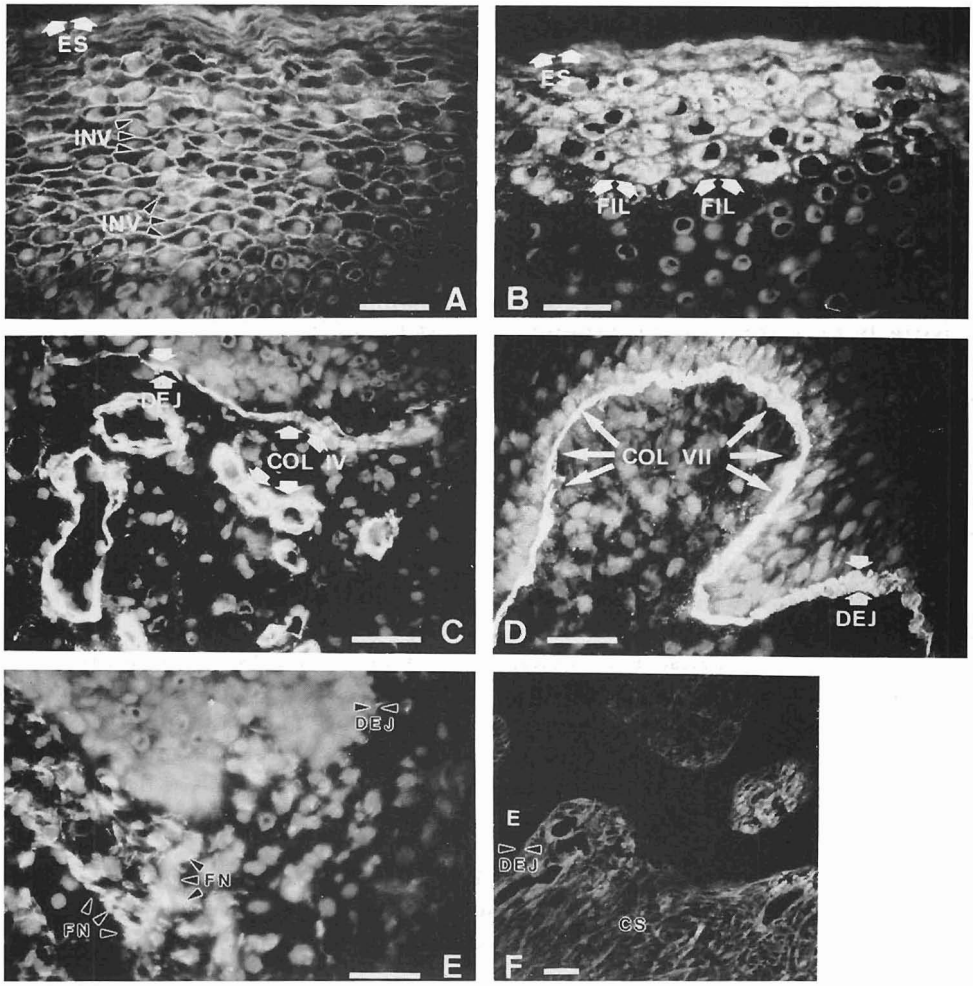

FIG. 4. Immunofluorescence microscopy of clinical biopsies at 16 days after grafting for markers of native skin. Skin antigens were studied by immunohistochemistry to compare skin regenerated from cultured skin grafts to ungrafted skin from cadaver donors as controls for antigen distribution (see Table II). Cell nuclei are labeled with propidium iodide for reference. (*A*) Monoclonal antibodies to involucrin (*INV*) label plasma membranes of all suprabasal keratinocytes extending to the epidermal surface (*ES*) of the biopsy; (*B*) Granular staining for filaggrin (*FIL*) is distributed in uppermost epidermal strata only and extends to the epidermal surface (*ES*); (*C*) Antibodies to collagen IV (*COL IV*) of basement membrane lamina densa produce specific staining of the dermal-epidermal junction (*DEJ*) and blood vessels (laminin identical, not shown); (*D*) Collagen VII (*COL VII*) antibodies identify anchoring fibrils of DEJ, but not blood vessels as distinguished from collagen IV; (*E*) Antibodies to fibronectin (*FN*) show filamentous staining in connective tissue subjacent to the DEJ; (*F*) Staining of biopsy from cultured skin with antibodies to chondroitin-sulfate (*CS*) showed uniform distribution of CS in healing connective tissue. Scale bars = 0.1 mm.

and then were stained with avidin-fluorescein (AV-FITC). After specific staining with fluorescein, cell nuclei were stained with 500 ng/ml propidium iodide. Samples were examined by epifluorescence microscopy.

RESULTS

Histology of Cultured Skin Grafts

Figure 1 shows the histologic appearance of the type of cultured cell-biopolymer skin analogue used in this study. Autologous epidermal keratinocytes and dermal fibroblasts are inoculated onto implantable collagen and GAG substrates. The epithelial component develops enucleate squamous outer layers, but keratinocytes attached to the substrate remain nucleated and cuboidal. The collagen-GAG material acts both as a culture surface and as a movable substrate for delivery of cells to wounds without disruption of cell-substrate attachments at the time of surgery.

Patient Photographs

Epithelialization of greater than half of the grafted area (marked with asterisks) has occurred by 10 days after grafting (Fig. 2, *top*). The remaining open areas develop islands of keratinized epithelium that progress radially and coalesce to close the wound. By 6 weeks after grafting (Fig. 2, *bottom*), the grafted site had a smooth external surface that was not raised compared to surrounding tissue, but was somewhat erythematous relative to uninjured skin. After reformation, the epidermis does not blister, and has good mechanical strength against lateral and perpendicular forces of moderate magnitude.

Histology at 2 Weeks

Histologic evaluation of the developing tissue at 14 days after grafting shows the highly interdigitated DEJ that is characteristic of this material at this timepoint (Fig. 3, *top*). Also at 2 weeks, the epidermis is somewhat hypertrophic, and the implanted collagen-GAG substrate is identifiable. Figure 3 (*bottom*) shows the distinctive reticulations of the collagen-GAG implant are associated with small numbers of leukocytes, but the immune response is not a dense infiltrate. Three months after application of cultured skin grafts, two of these three patients had biopsies collected (Table I), and both had flat dermal-epidermal junctions, resolution of epidermal hypertrophy, and stable connective tissue under the epidermis (not shown).

Immunohistochemistry of Epidermal Differentiation, DEJ, and dermis

At 16 days after grafting, all seven markers of human skin that were investigated were found to be expressed. Markers of epidermal differentiation, involucrin (Fig. 4*A*) and filaggrin (Fig. 4*B*), were expressed in suprabasal keratinocytes, but more than in the native skin control (Table II). Lamellar bodies representing epidermal barrier function are also present by 2 weeks after grafting (not shown).

More importantly, components of basement membrane, collagen type IV (Fig. 4*C*) and laminin (identical to collagen IV, see Table II), stain intensely and specifically at both the DEJ and around blood vessels. These distributions are homologous with native skin control (Table II). By

TABLE II

Expression of Skin Antigens in Split-Thickness Human Skin, and Biopsy Skin from Patient 3 Collected 16 Days after Treatment with a Cultured Skin Graft

	Skin Compartment						
	Epidermal Stratum				DEJ	Dermis	
Antigen	Corneum	Granulosum	Spinosum	Germinativum		Papillary	Reticular
Involucrin*							
CSG	3	3	3	0	N/A	N/A	N/A
STHS	4	2	0	0	N/A	N/A	N/A
Filaggrin*							
CSG	3	3	0	0	N/A	N/A	N/A
STHS	2	4	0	0	N/A	N/A	N/A
Collagen IV†							
CSG	0	0	0	0	4	4	4
STHS	0	0	0	0	4	4	4
Laminin‡							
CSG	0	0	0	0	4	4	4
STHS	0	0	0	0	4	4	4
Collagen VII§							
CSG	0	0	0	2	4	1	0
STHS	0	0	0	0	4	0	0
Chondroitin-SO_4¶							
CSG	N/A	N/A	N/A	N/A	0	3	3
STHS	N/A	N/A	N/A	N/A	0	4	2
Fibronectin†							
CSG	N/A	N/A	N/A	N/A	0	2	1
STHS	N/A	N/A	N/A	N/A	0	4	2

Ordinal scale: 0–4; 0 = none, 4 = most; N/A, not applicable.

* Biomedical Technologies, Stoughton, Mass.

† Biodesign, Inc., Kennebunkport, Maine.

‡ Telios Pharmaceutical, San Diego, Calif.

§ Lynn Y. Sakai, Ph.D., Shriners Hospitals for Crippled Children, Portland, Oreg.

¶ J. Michael Sorrell, Ph.D., Case Western Reserve University, Cleveland, Ohio.

Abbreviations: DEJ, dermal-epidermal junction; CSG, cultured skin graft; STHS, split-thickness human skin (control, ungrafted).

16 days after grafting, continuous and intense staining for collagen VII (Fig. 4*D*) accounts for absence of blistering of regenerated epidermis. The epidermis is both well interdigitated and biologically adhered to connective tissue. Collagen VII identifies the DEJ, but not the vascular plexus in regenerated connective tissue.

Markers of normal human dermis, fibronectin (Fig. 4*E*), and chondroitin-sulfate (Fig. 4*F*) are also found in the healing connective tissue at 16 days after grafting. Although the distributions of these markers are not identical to control skin (Table II), their expression suggests that multiple components of dermis develop in new connective tissue.

Anchoring Fibrils after 2 Weeks

Anchoring fibrils at 2 weeks occur frequently, but are not highly organized, as shown in Figure 5. Together with the results of immunohistochemistry of collagen type VII, this suggests that formation and development of anchoring fibrils is continuing at this timepoint. By comparison, after 3 months, dense and continuous distributions of anchoring fibrils (not shown) account for the clinical observations that epidermal blistering does not occur with this kind of cultured skin graft.

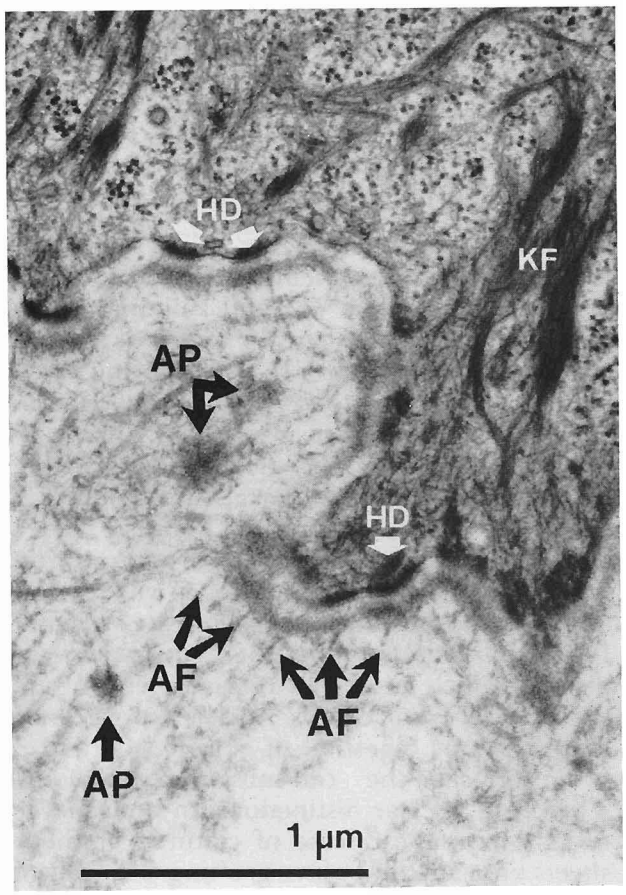

FIG. 5. Transmission electron microscopy of cultured skin biopsies showing DEJ, anchoring fibrils (*AF*) and anchoring plaques (*AP*) at 2 weeks after grafting of cultured skin grafts. Two weeks after grafting, anchoring fibrils and anchoring plaques seem incompletely formed and in relatively low frequency, but account for the absence of epithelial blistering after healing. Scale bar = 1 μm.

Collagen-GAG Degradation at 2 Weeks, New Collagen at 3 Months

Metabolic clearance of the biopolymer implant at 2 weeks results from degradative actions of macrophages, lymphocytes, and granulocytes on the biopolymer implant, as shown in Figure 6. Macrophages actively phagocytize the collagen-GAG biopolymer implant, whereas adjacent fibroblasts are exposed to paracrine cytokines from macrophages that stimulate collagen synthesis and deposition. Lymphocytes are associated with biopolymer implants by 2 weeks, but the condensed chromatin in the nucleus of this cells suggests it is not activated.

Replacement of the biopolymer implant with new fibrous collagen at 3 months after grafting is performed by fibroblasts that arrive in the site either by transplantation or by migration from the woundbed. Figure 7 shows that similar to native dermis, the new collagen formed in the connective tissue by 3 months is distributed in orthogonal array, or a "basketweave" pattern. This pattern of collagen is easily distinguished from the highly aligned and parallel collagen bundles of scar.

DISCUSSION

Data presented here support the hypothesis that rapid closure of skin wounds can be accomplished with a composite material fabricated from autologous epidermal and dermal skin cells attached to an implantable, collagen-based substrate. Biologic attachment of epidermal cells to the substrate allows: (1) easy handling and delivery of cells for grafting; (2) elimination of enzymatic treatment of epidermal cell sheets at the time of surgery; and (3) application of epithelial and mesenchymal cells in a single operative procedure in analogy to split-thickness skin. This cultured cell-biopolymer model of skin satisfies many of the criteria for skin substitutes described by Pruitt and Levine,[27] although certain factors, including cost effectiveness and outcome, are not considered here.

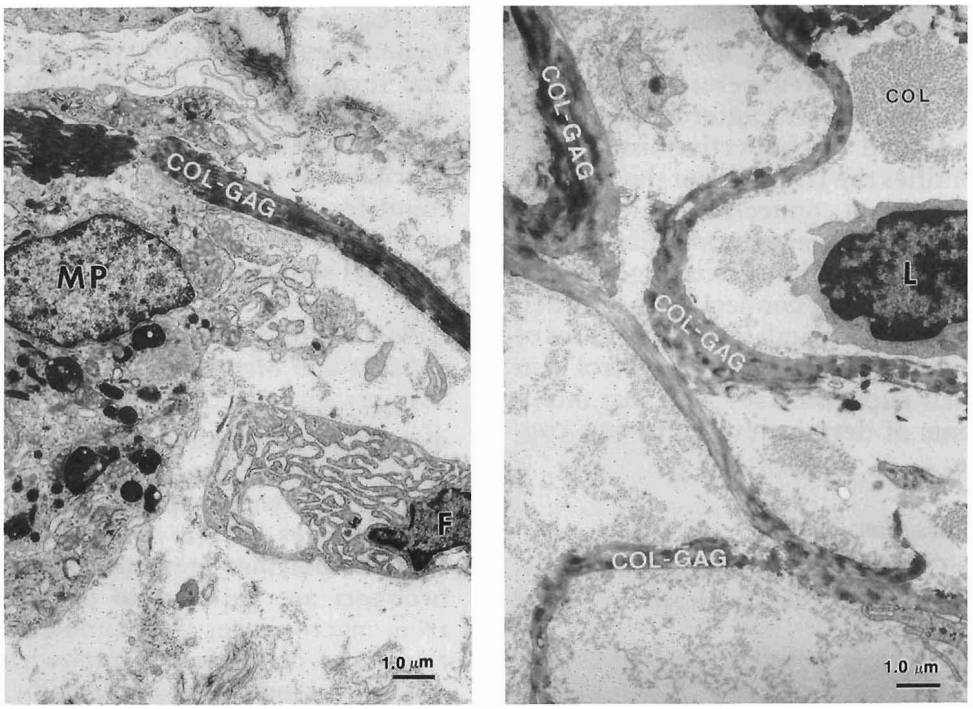

FIG. 6. Degradation of implanted collagen-GAG at 2 weeks in wounds treated with cultured skin grafts. *Left panel*: Collagen-GAG is phagocytized by a macrophage (*MP*) proximal to a fibroblast (*F*) in regenerating connective tissue. *Right panel*: Reticulations of collagen-GAG are adjacent to a lymphocyte (*L*) and new collagen (*COL*). Scale bars = 1 μm.

Combination of cultured skin cells with a collagen-GAG substrate utilizes principles described by Yannas and Burke,[28,29] but with important distinctions. The Yannas model consists of an acellular collagen-GAG sponge 2 to 3 mm thick covered on one side with silicone rubber as a temporary barrier to fluid loss and infection. Clinically, it is applied to excised wounds, allowed to vascularize, and then the silicone barrier is replaced with a thin epidermal autograft in a second operative procedure. Because of its thickness and pore structure, that material is not suitable for simultaneous application of epithelial cells in place of the silicone layer. In contrast, single application of stratified epithelium on a collagen-GAG substrate requires a total thickness of the graft no greater than 0.5 mm.[16,17] This allows survival of the epithelium by diapedesis during vascularization of the substrate. Use of cultured epithelium removes the limitation imposed by donor site availability that is required by an acellular implant.

Culture of epidermal keratinocytes on a graftable substrate allows initiation in vitro of the complex system of structural proteins known as the DEJ. The DEJ extends from vinculin and talin as the intracellular binding sites for actin and intermediate filaments of keratinocytes through the plasma membrane, through integrins,[30,31] to laminin[21,32] and kalinin[33] of the lamina lucida, to collagens IV and V of the lamina densa, to collagen VII of anchoring fibrils and anchoring plaques,[20,34] to fibronectin and collagens I and III in dermis.[22] This complex of proteins constitutes the mechanical bond between epidermis and dermis, and must be present to restore the strength of native epidermal tissue. Most of these proteins are known to be synthesized by keratinocytes in vitro,[21,31,35] and are deposited at the cell-substrate interface. It is also understood that the neutral protease, dispase, which is used to release sheets of epithelial cells from culture vessels before grafting,[4,5] acts by digestion of collagen IV and fibronectin at the cell-substrate junction.[36] Therefore, a clear distinction can be made between enzymatic release of cultured epithelial sheets immediately before grafting and delivery of cultured epithelial sheets on a dermal analogue with cell-substrate attachments intact. Elimination of the enzymatic treatment of cultured epithelial sheets is concluded to facilitate reformation of functional DEJ, contribute to improved epidermal strength, and account for the

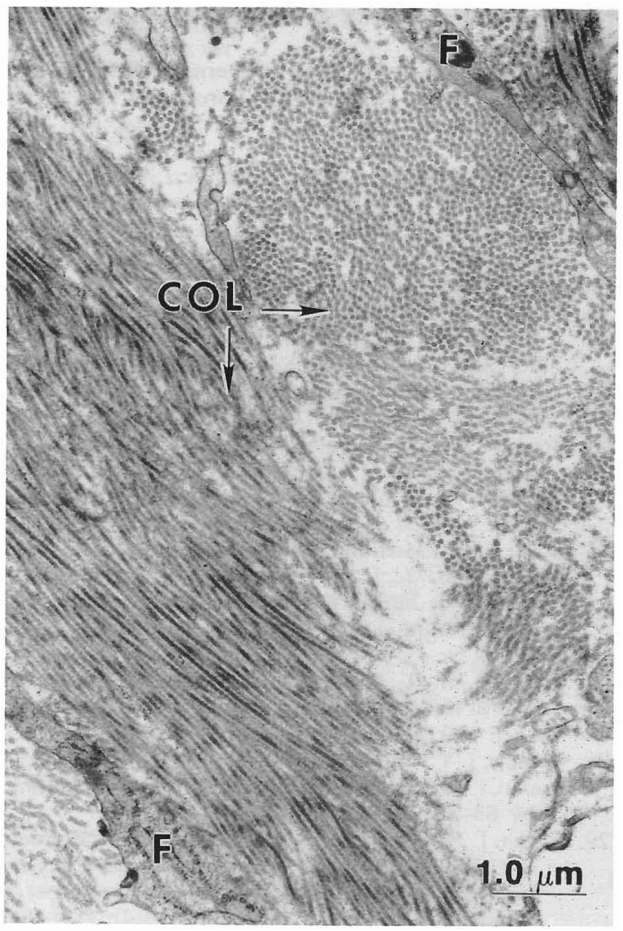

FIG. 7. Orthogonal distribution of new collagen at 3 months. Collagen (*COL*) deposited by fibroblasts (*F*) is distributed in orthogonal, or "basketweave" array in analogy to native dermis, and in contrast to parallel collagen of scar. Scale bar = 1 μm.

absence of blistering in epidermal tissue reformed from composite grafts as supported indirectly by the present study.

Although epidermal tissue regeneration has been demonstrated and is now available commercially, reports of dermal tissue regeneration on burn wounds are few.[8–10] The presence of degrading collagen-GAG implant in a noninflamed wound at 2 weeks after grafting demonstrates the biocompatibility of the biopolymer material. The absence of the implant in biopsies at 4 weeks after grafting suggests that it is completely degraded within 1 month, which is in agreement with previous studies.[9,24]

Current limitation of broad utilization of this model for burns and plastic surgery results from highly variable initial engraftment (15–65 percent) that can be attributed to deficiencies in: (1) optimal keratinization and viability of epithelium in the cultured graft; (2) woundbed vascularity by pretreatment with allograft skin versus fresh excision to subcutaneous fat; (3) microbial contamination that destroys cultured skin; and (4) choice and administration of antimicrobial drugs on the recipient site during the first 4 to 7 days after grafting.[37,38] Solutions to the limitation of variable engraftment are expected to result from improved biologic homology of composite cultured skin grafts with split-thickness skin, rapid and uniform angiogenesis into the dermal component of the cultured graft, and delivery to the graft site of antimicrobial drugs with low toxicity to cultured cells and high toxicity to burn or skin wound microorganisms. With rates of engraftment that approach split-thickness skin (>90 percent), the advantages of reduced donor sites, fewer surgical procedures, and shorter hospitalization after major burns may be realized. Skin anatomy and expression of histiotypic markers of epidermis, DEJ, and dermis described in this report provide evidence that functional full-thickness skin can be restored with composite cultured grafts applied to excised burns in a single procedure.

Steven Boyce, Ph.D.
Department of Surgery; ML 558
University of Cincinnati Medical Center
231 Bethesda Avenue
Cincinnati, Ohio 45267–0558

REFERENCES

1. Tanner, J. C., Vandeput, J., and Olley, J. F. The mesh skin autograft. *Plast. Reconstr. Surg.* 34: 287–292, 1964.
2. Warden, G. D., Saffle, J. R., and Kravitz, M. A two-stage technique for excision and grafting of burn wounds. *J. Trauma* 22: 98–103, 1982.
3. Housinger, T. A., Warden, G. D., Lang, D., Visse, M., and Pohlman, S. The use of sheet skin grafts in pediatric burn patients. *Proc. Am. Burn Assoc.* 23: 32, 1991.
4. Green, H., Kehinde, O., and Thomas, J. Growth of human epidermal cell into multiple epithelia suitable for grafting. *Proc. Natl. Acad. Sci. USA* 76: 5665–5668, 1979.
5. Gallico, G. G. III, O'Conner, N. E., Compton, C. C., Kehinde, O., and Green, H. Permanent coverage of large burn wounds with autologous cultured human epithelium. *N. Engl. J. Med.* 311(7): 448–451, 1984.
6. Heck, E. L., Bergstresser, P. R., and Baxter, C. R. Composite skin graft: Frozen dermal allografts support the engraftment and expansion of autologous epidermis. *J. Trauma* 25(2): 106–112, 1985.
7. Cuono, C. B., Langdon, R., Birchall, N., Barttelbort, S., and McGuire, J. Composite autologous-allogeneic skin replacement: Development and clinical ap-

plication. *Plast. Reconstr. Surg.* 80(4): 626–635, 1987.
8. Heimbach, D., Luterman, A., Burke, J. F., Cram, A., Herndon, D., Hunt, J., Jordan, M., McManus, W., Solem, L., Warden, G., and Zawacki, B. Artificial dermis for major burns; a multi-center randomized clinical trial. *Ann. Surg.* 208: 313–320, 1988.
9. Hansbrough, J. F., Boyce, S. T., Cooper, M. L., and Foreman, T. J. Burn wound closure with cultured human keratinocytes and fibroblasts attached to a collagen-glycosaminoglycan substrate. *JAMA* 262: 2125–2130, 1989.
10. **Hull, B. E., Finley, R. K., and Miller, S. F.** Coverage of burns with bilayered skin equivalents: a preliminary clinical trial. *Surgery* 107(5): 496–502, 1990.
11. Compton, C. C., Gill, J. M., Bradford, D. A., Regaur, S., Gallico, G. G. III, and O'Connor, N. E. Skin regenerated from cultured epithelial autografts on full-thickness burn wounds from 6 days to 5 years after grafting. A light, electron microscopic and immunohistochemical study. *Lab. Invest.* 60(5): 600–612, 1989.
12. Munster, A. M., Weiner, S. H., and Spence, R. J. Cultured epidermis for the coverage of massive burn wounds. A single center experience. *Ann. Surg.* 211(6): 676–680, 1990.
13. Woodley, D. T., Peterson, H. D., Herzog, S. R., Stricklin, G. P., Burgeson, R. E., Briggaman, R. A., Cronce, D. J., and O'Keefe, E. J. Burn wounds resurfaced by cultured epidermal autografts show abnormal reconstitution of anchoring fibrils. *JAMA* 259: 2566–2571, 1988.
14. **Boyce, S. T., and Ham, R. G.** Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J. Invest. Dermatol.* 81(1), supplement: 33s–40s, 1983.
15. **Boyce, S. T., and Ham, R. G.** Cultivation, frozen storage, and clonal growth of normal human epidermal keratinocytes in serum-free media. *J. Tissue Cult. Methods* 9(2): 83–93, 1985.
16. Boyce, S. T., Christianson, D. J., and Hansbrough, J. F. Structure of a collagen-GAG dermal skin substitute optimized for cultured human epidermal keratinocytes. *J. Biomed. Materials Res.* 22(10): 939–957, 1988.
17. **Boyce, S. T., and Hansbrough, J. F.** Biologic attachment, growth and differentiation of human epidermal keratinocytes onto a graftable collagen and chondroitin-6-sulfate substrate. *Surgery* 103(4): 421–431, 1988.
18. Murphy, G. F., Flynn, T. C., Rice, R. H., and Pinkus, G. S. Involucrin expression in normal and neoplastic human skin: A marker for keratinocyte differentiation. *J. Invest. Dermatol.* 82(5): 453–457, 1984.
19. Dale, B. A., Resing, K. A., and Lonsdale-Eccles, J. D. Filaggrin: A keratin filament associated protein. *Ann. N.Y. Acad. Sci.* 455: 330–342, 1985.
20. Sakai, L. Y., Keene, D. R., Morris, N. P., and Burgeson, R. E. Type VII collagen is a major structural component of anchoring fibrils. *J. Cell Biol.* 103: 1577–1586, 1986.
21. Prunieras, M., Regnier, M., Fougere, S., and Woodley, D. Keratinocytes synthesize basal-lamina proteins in culture. *J. Invest. Dermatol.* 81(1): Suppl. 1, 74s–81s, 1983.
22. Mosher, D. F., Fogerty, F. J., Chernousov, M. A., and Barry, E. L. Assembly of fibronectin into extracellular matrix. *Ann. N.Y. Acad. Sci.* 614: 167–180, 1991.
23. Sorrell, J. M., Caterson, B., Caplan, A. I., Davis, B., and Schafer, I. A. Human keratinocytes contain carbohydrates that are recognized by keratan sulfate-specific monoclonal antibodies. *J. Invest. Dermatol.* 95(3): 347–352, 1990.
24. Boyce, S. T., Foreman, T. J., English, K. B., Stayner, N., Cooper, M. L., Sakabu, S., and Hansbrough, J. F. Skin wound closure in athymic mice with cultured human cells, biopolymers and growth factors. *Surgery* 110(5): 866–876, 1991.
25. **Boyce, S. T., and Williams, M. L.** Free fatty acids and vitamin E induce lamellar bodies and precursors of barrier lipids in lifted dermal-epidermal skin substitutes. *J. Invest. Dermatol.* 96(4): 620A, 1991.
26. Boyce, S. T., Medrano, E. E., Abdel-Malek, Z. A., and Nordlund, J. J. Pigmentation of cultured epithelium by human melanocytes in a composite skin substitute model. *J. Cell Biol.* 115(3,pt2): 357a, 1991.
27. **Pruitt, B. A. Jr., and Levine, S.** Characteristics and uses of biologic dressings and skin substitutes. *Arch. Surg.* 119: 312–322, 1984.
28. **Yannas, I. V., and Burke, J. F.** Design of an artificial skin. I. Basic design principles. *J. Biomed. Material Res.* 14: 65–81, 1980.
29. Burke, J. F., Yannas, I. V., Quinby, W. C. Jr., Bondoc, **C. C., and Jung, W. K.** Successful use of a physiologically acceptable artificial skin in the treatment of extensive burn injury. *Ann. Surg.* 194(4): 413–428, 1981.
30. Stepp, M. A., Spurr-Michaud, S., Tisdale, A., Elwell, J., and Gipson, I. K. $\alpha 6$-$\beta 4$ integrin heterodimer is a component of hemidesmosomes. *Proc. Natl. Acad. Sci. USA* 87(22): 8970–8974, 1990.
31. Marchisio, P. C., Bondanza, S., Cremona, O., **Cancedda, R., DeLuca, M.** Polarized expression of integrin receptors ($\alpha 6$-$\beta 4$, $\alpha 2$-$\beta 1$, $\alpha 3$-$\beta 1$ and $\alpha 3$-$\beta 5$) and their relationship with cytoskeleton and basement membrane matrix in cultured human keratinocytes. *J. Cell Biol.* 112(4): 761–773, 1991.
32. Gehlsen, K. R., Dillner, L., Engvall, E., and Ruoslahti, E. The human laminin receptor is a member of the integrin family of cell adhesion receptors. *Science* 241(4870): 1228–1229, 1988.
33. Rousselle, P., Lundstrum, G. P., Keene, D. R., and **Burgeson, R. E.** Kalinin: An epithelium-specific basement membrane adhesion molecule that is a component of anchoring fibrils. *J. Cell Biol.* 114(3): 567–576, 1991.
34. Smith, L. T., Sakai, L. Y., Burgeson, R. E., Holbrook, **K. A.** Ontogeny of structural components at the dermal-epidermal junction in human embryonic and fetal skin: The appearance of anchoring fibrils and type VII collagen. *J. Invest. Dermatol.* 90(4): 480–485, 1988.
35. O'Keefe, E. J., Woodley, D. T., Falk, R. J., Gammon, W. **R., and Briggaman, R. A.** Production of fibronectin by epithelium in a skin equivalent. *J. Invest. Dermatol.* 88(5): 634–639, 1987.

36. Stenn, K. S., Link, R., Moellman, G., Madri, J., Kuklinska, E. Dispase, a neutral protease from Bacillus polymyxa, is a powerful fibronectinase and type IV collagenase. *J. Invest. Dermatol.* 93(2): 287–290, 1989.
37. Cooper, M. L., Boyce, S. T., Hansbrough, J. F., Foreman, T., and Frank, D. Cytotoxicity to cultured human keratinocytes (HK) of topical antimicrobial agents. *J. Surg. Res.* 48(3): 190–195, 1990.
38. McCauley, R. L., Linares, H. A., Pelligrini, V., Herndon, D. N., Robson, M. C., and Heggers, J. P. In vitro toxicity testing of topical antimicrobial drugs to human fibroblasts. *J. Surg. Res.* 46(3): 267–74, 1989.