Skin wound closure in athymic mice with cultured human cells, biopolymers, and growth factors

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Skin wound closure remains a major problem in acute and reconstructive skin grafting after large burns because of limited availability of donor skin. This report evaluates six protocols for preparation in vitro of skin substitutes composed of cultured human cells. biopolymers, and growth factors for wound closure. Full-thickness wounds in athymic mice treated in a single procedure with cultured skin substitutes were compared directly to treatments with murine skin autograft, human skin xenograft, or no graft. Rectilinear planimetry of healed wounds 6 weeks after surgery showed that skin substitutes cultured in serum-free medium, and for 24 hours before surgery in defined medium with basic fibroblast growth factor (100 ng/ml), were not statistically different (p < 0.05) in size from treatment with human skin xenograft. Acceptance and persistence of skin substitutes cultured in serum-free media were 70% at 6 weeks after surgery, as determined by staining of healed skin with a fluorescein-labeled monoclonal antibody against human HLA-ABC antigens. Ultrastructural examination of wounds with cultured human skin 6 weeks after treatment showed complete basement membrane, including anchoring fibrils, presence of melanocytes and pigment transfer to keratinocytes, and innervation of healed skin adjacent to basement membrane. These findings demonstrate effectiveness of cultured skin substitutes for closure of skin wounds and illustrate important capabilities to modulate the natural processes of wound repair, to increase supply of materials used for wound repair, and to enhance quality of wound healing. (SURGERY 1991;110:866-76.)

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UNINJURED TISSUES AND ORGANS of the body are composed of cells, extracellular matrices of biopolymers, and regulatory molecules that affect cell, tissue, and organ function. Noncongenital injury to cells and tissues causes wounds and initiates common mechanisms of wound repair at all sites in the body.¹ Fundamental components of wound closure include restoration of stable ectoderm-derived tissue (epithelium or endothelium) and of uniform vascular supply in the adjacent meso-

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Reprint requests: Steven Boyce, PhD, Department of Surgery; ML 558, University of Cincinnati Medical Center, 231 Bethesda Ave., Cincinnati, OH 45267-0558. 11/56/26556 derm-derived tissue. For optimal closure, wounds caused by traumatic injury or elective surgery require rapid restoration of normal tissue anatomy in the absence of infection.² This study describes closure of full-thickness skin wounds, but principles of increased availability and synthesis of biologic materials apply to intervention in repair of any wound.

Skin substitutes for closure of full-thickness skin wounds have been prepared from in vitro³⁻⁶ or ex vivo^{7, 8} sources. In vitro substitutes for human skin, which are composed of cultured keratinocyte and fibroblast cells,⁹⁻¹⁴ biopolymers,^{4, 5, 15-18} and growth factors,¹⁹⁻²¹ can accomplish wound closure as shown in animal^{4, 22-25} and clinical^{7, 8, 26-31} studies. However, quantitative data that compare the functional outcome (percentage of original wound size and percentage of graft acceptance) of skin substitutes to native skin are lacking

in these previous studies. This report contains quantitative comparison to split-thickness human skin of two types of skin substitutes^{3, 4} prepared from collagenbased matrices that are populated internally with cultured human fibroblasts (HF) and populated externally with cultured human keratinocytes (HK). Closure of skin wounds is also shown to be influenced beneficially by exposure of skin substitutes to basic fibroblast growth factor (bFGF).³² Beneficial effects of bFGF require that skin substitutes containing HK are cultured in nutrient media without fetal bovine serum (FBS). Basic FGF is a multipotent mitogen that can stimulate angiogenesis³³ in vivo and growth of HK in vitro in biochemically defined medium.³⁴⁻³⁶ The findings of this report suggest that preincubation of skin substitutes in bFGF simultaneously promotes the two essential components of skin wound closure, vascularization and epithelialization.

METHODS

Experimental treatments. All animal studies were approved by the University of California San Diego Animal Subjects Committee. Cultured skin substitutes were compared on athymic mice (BALB/c, nu/nu) to no graft (treatment A; n = 18), human xenograft (treatment H; n = 17), or murine autograft (treatment I; skin rotated 180 degrees; n = 16). Mice were anesthetized with tribromoethanol in tertiary amyl alcohol (Avertin), and 2×2 cm full-thickness skin defects were prepared to a depth leaving the panniculus carnosus intact. Wound perimeters were tattooed at eight points; treatments were administered; nonadherent wound dressing (N-Terface; Winfield Laboratories, Richardson, Texas) was placed over the grafts; and stent-type sutures secured the N-Terface and grafts to the wound margin and wound bed. Grafts were dressed with Xeroform (Sherwood Laboratories, St. Louis, Mo.) and cotton gauze; sutures were tied over the dressings and covered with adhesive bandages. Data were collected 6 weeks after surgery.

Components of skin substitutes. Cultured skin substitutes were prepared from separate cultures of HK, HF, acellular collagen-glycosaminoglycan (C-GAG) membranes, or HK plus collagen gels containing HF (C-GEL-HF). C-GAG membranes were made as previously described.¹⁶ C-GEL-HF were prepared by Dr. Kathleen English at the University of Utah, Salt Lake City, Utah, according to Bell et al.^{4, 5} and were sent by express delivery to the University of California San Diego Medical Center. Culture media used to prepare cultured skin substitutes are summarized in Table I: (a) Dulbecco's modified Eagle's medium supplemented with 10% FBS, 10 ng/ml epidermal growth factor, 5

Table I. Media compositions

Medium designation	а	Ь	С							
Basal medium	DMEM	MCDB 153	MCDB 153							
Defined supplements										
bFGF (ng/ml)			10-100							
EGF (ng/ml)	10	10								
Insulin $(\mu g/ml)$	5	5	5							
Hydrocortisone	0.5	0.5	0.5							
$(\mu g/ml)$										
Undefined supplements										
BPE (vol/vol)		0.5%								
FBS (vol/vol)	10%									

 μ g/ml insulin, and 0.5 μ g/ml hydrocortisone; (b) serum-free MCDB 153^{12, 13} containing 0.5% (vol/vol) bovine pituitary extract, with elevated concentrations of selected amino acids,²⁸ and (c) biochemically defined MCDB 153 in which the epidermal growth factor is replaced with 10 to 100 ng/ml bFGF.^{37, 38}

Preparation of skin substitutes. Six protocols for combination of cultured cells, biopolymer matrices, and culture media were used to prepare skin substitutes and to test for their effects on wound contraction and graft acceptance. Preparation protocols are summarized in Table II. Skin substitutes were made with lyophilized, cross-linked C-GAG (treatments B to E and G) or with hydrated C-GEL-HF polymer matrices (treatment F). Cultured human skin cells (HF or HK) were added (+) or not added (-). HF were inoculated onto the porous surface of the C-GAG sponge, and HK inoculated onto the nonporous film was added to one surface of the C-GAG substrate.¹⁶ Cells were inoculated onto matrices during sequential incubations (i, ii, iii). Media (a, b, c; Table I) and time (days) of incubations were performed as indicated in Table II. Growth factors in serum-free or defined media were added as indicated. Basic FGF was added to media for treatments C and D at 10 ng/ml after purification from bovine pituitaries (Dr. Gary Shipley, Oregon Health Sciences University, Portland, Ore.) and for treatment G at 100 ng/ml of the human recombinant gene product (Synergen, Inc., Boulder, Colo.).

Data collection and analysis. Data were collected at 6 weeks after surgery for analysis of wound contraction and graft acceptance. Wound contraction was determined by rectilinear planimetry of areas bounded by tatoo marks made at the wound margin at the time of surgery. Data for wound contraction are expressed as percentage of original wound size (mean \pm SEM).³⁹ Data from multiple test groups in wound contraction



Fig. 1. Histology of cultured skin substitute and of split-thickness skin. *Top panel*, Cultured skin substitute composed of a C-GAG substrate populated internally with cultured HF and externally with cultured HK. *Bottom panel*, Human split-thickness skin graft. Scale bars = 0.1 mm.

Treatment	N	Matrix	Cell and incubation		Incubations (medium/days)			Supplements for serum-free media	
			HF	HK	i	ii	iii	EGF, BPE	bFGF
А	18	None	_		None	None	None	_	_
В	9	C-GAG	_	+	b/3	a/3	None	+	-
С	9	C-GAG	_	+	b/3	a/3	c/0.25	+	+
D	9	C-GAG	+	+	b/3	a/3	c/0.25	+	+
E	9	C-GAG	+	+	b/3	a/3	None	+	_
F	7	C-GEL	+	+	a/5	b/3	None	+	_
G	10	C-GAG	+	+	a/3	b/3	c/1	+	+
Н	17	Human xe	nograft						
I	16	Murine au	tograft						

Table II. Preparation protocols

i, First incubation; ii, second incubation; iii, third incubation; a, medium a; b, medium b; c, medium c from Table I. (i, ii, iii, Sequential incubations of HF and/or HK cells, on C-GAG or C-GEL matrix in media "a", "b", or "c" for 0.25 to 5 days per incubation.)

11K, Human epidermal keratinocytes; 11F, human dermal fibroblasts.

studies were subjected to analysis of variance. Honest significant difference between pairs of treatment conditions was determined by Tukey's Studentized Range Test at a 95% level of confidence (p < 0.05). Graft acceptance was determined by direct immunofluorescence staining of healed epidermis with fluorescein-labeled monoclonal antibody against a common hapten of the HLA-ABC histocompatibility antigens⁴⁰ (Accurate Chemical and Scientific Corp., San Diego, Calif.). Tissue samples for immunohistochemical staining were prepared from cryostal sections of unfixed, fresh-frozen excised skin from wound sites. Data for graft acceptance



Fig. 2. External and light microscopic appearances of healed wounds on athymic mice after treatment of fullthickness skin defects with cultured skin substitutes. A, Wound closed with C-GAG, HF, HK skin substitute (treatment G) shows reduced wound contraction and pigmented spots (arrows) generated from transplanted human melanocytes. B, Wound closed with C-GEL, HF, HK skin substitute (treatment F) shows reduced wound contraction and pigmented spots (arrows). C, Light micrograph of skin regenerated from C-GAG, HF, HK skin substitute shows resorbance of implanted C-GAG substrate (arrowheads). D, Light micrograph of skin regenerated from C-GEL, HF, HK skin substitute (Scale bars C, D = 0.1 mm). E, No graft (treatment A). F, Human split-thickness skin (treatment H). G, Murine autograft (treatment I).

are expressed as percentage of HLA-ABC positive wounds and were subjected to chi-squared analysis. Microscopic (light and electron) analyses were performed by standard methods.

RESULTS

Histology of composite skin substitute and of natural human skin. Fig. 1 shows representative histology of composite skin substitute composed of C-GAG substrate combined with HK and HF (Fig. 1, top panel) and is compared to split-thickness human skin (Fig. 1, bottom panel). Total thickness (<1 mm) and histologic organization of the skin substitute is very similar to split-thickness skin with the epithelial component restricted to the external surface. The reticulated surfaces of the C-GAG substrate appear in transverse section as a darkly stained network between open pores in the substrate. HF move into the C-GAG substrate after inoculation, but are located more frequently towards the external surface of the C-GAG sponge.

Closed wounds on athymic mice at 6 weeks after surgery. Wounds treated with human or murine skin (treatments H and I, respectively) closed most rapidly by subjective evaluation with complete and stable closure occurring by 2 weeks after surgery. Although time required for wound closure was not quantitated,



Fig. 3. Plot of percentage of original wound size versus wound treatment at 6 weeks after surgery. Treatment A, no graft; treatments B to G, cultured skin substitutes as detailed in Table II; treatment H, human split-thickness skin xenograft; treatment I, murine autograft. *Treatment is significantly different (p < 0.05) from human xenograft control. **Treatment is not significantly different (p < 0.05) from human xenograft control.

wounds treated with skin substitutes (treatments B to G) appeared to require 2 to 4 weeks to close completely and to develop fully keratinized and desquamating epidermal surfaces. Fig. 2 shows demarcated wound sites 6 weeks after surgery. Skin substitutes made from C-GAG, cultured human cells, and growth factors (Fig. 2, A; treatment G) result in closure of wounds with a rectangular wound perimeter. Initial indication of the persistence of human tissue on the closed wound is the presence of pigmented spots within the demarcated wound perimeter on this albino animal. Histologic examination of wounds closed by treatment G (Fig. 2, C) shows a fully differentiated and desquamating epidermis attached to regenerating connective tissue between the epidermis and panniculus carnosus. Wounds closed with C-GEL and cultured human cells (Fig. 2, B; treatment F) also show wound perimeters that are straight rather than curved and infrequently occurring pigment spots on markedly hypopigmented tissue. Regenerated tissue in the wound perimeter also histologically shows a fully differentiated and desquamating epidermis attached to connective tissue over the panniculus carnosus (Fig. 2, D). Negative controls (treatment A) received no graft (Fig. 2, E), and wounds closed by pronounced contraction. Epidermal migration from the wound perimeter and wound contraction produced typical curved wound margins. Application of split-thickness human skin (Fig. 2, F; treatment H) results in complete wound closure in 2 to 3 weeks. Hyperpigmentation of natural human skin on athymic mice is typical and provides indirect confirmation of the acceptance and persistence of human skin on the wounds. Human epidermis on the mouse is usually thickened and often hyperkeratotic, compared to native murine epidermis. Murine autograft (treatment I) also results in rapid and complete wound closure (Fig. 2, G). Wounds closed with autograft show histologic organization comparable to native murine skin and no hyperpigmentation.

Wound contraction at 6 weeks after surgery. Quantitative data collected from rectilinear planimetery of healed wounds are summarized in Fig. 3. Wounds that received no graft (treatment A) contracted most, to $19.3\% \pm 1.2\%$ of original size. Wounds treated with C-GAG skin substitutes in which HK cells had been exposed to FBS (treatments B to E) all contracted to mean wound sizes of approximately 24% of original size and were not different statistically from the negative control (treatment A). Skin substitutes prepared from C-GEL-HF and HK (treatment F) were not statistically different from negative control or from positive control of human xenograft, with $33.0\% \pm 3.7\%$ original wound size. C-GAG-HF-HK skin substitutes in which the HK were not exposed to FBS and were exposed to bFGF (treatment G) showed the least contraction of any of the cultured skin substitutes, with $38.5\% \pm 2.6\%$ original wound size. This value was not different statistically from split-thickness human skin $(50.5\% \pm 5.6\%$ of the original size), but was different



Fig. 4. Direct immunofluorescence staining with fluorescein-labeled monoclonal antibody against a common hapten of the HLA-ABC antigens, 6 weeks after treatment of wounds with cultured skin substitutes or with split-thickness skin graft. Net-like pattern of staining is specific for a common hapten of the cell surface markers HLA-ABC. (Tissue also stained with propidium iodide which labels cell nuclei.) A, epidermis of human skin xenograft. B, epidermis and dermis C-GAG, HF, HK graft. C, epidermis at wound margin between C-GEL, HF, HK graft (epidermis labeled on right) and murine skin (epidermis not labeled on left). Scale bars = 0.1



Fig. 5. Plot of percentage of HLA-ABC positive wounds versus wound treatment at 6 weeks after surgery. Treatment E, C-GAG, HK, HF skin substitutes with HK exposed to serum before grafting, 33% graft acceptance. Treatment F, C-GEL, HF, HK skin substitutes with HK in serum-free medium before grafting, 71% graft acceptance. Treatment G, C-GAG, HF, HK skin substitutes with HK in serum-free medium before grafting, 70% graft acceptance. Treatment H, human skin xenograft, 94% acceptance.

statistically from no graft (treatment A). C-GAG-HF-HK skin substitutes also were not different statistically from C-GEL-HF-HK grafts, with respect to wound contraction. As expected, murine autograft (treatment I) contracted least of any wound treatment, with $66.4\% \pm 4.2\%$ original wound size at 6 weeks after surgery.

Acceptance of grafts containing human cells. Positive and specific staining with fluorescein-labeled monoclonal antibody against HLA-ABC antigens produces a distinctive net-like pattern of fluorescence in the epidermis. This pattern identifies the distribution of these antigens at the surfaces of human keratinocytes (Fig. 4). Specific staining for HLA-ABC of epidermis on closed wounds was scored as graft acceptance. Human skin xenograft stains specifically and uniformly for HLA-ABC (Fig. 4, A). Stratified epidermis develops from C-GAG-HF-HK grafts, and labeling of fibroblasts is also seen (Fig. 4, B). Similarly, acceptance of cultured epithelium of C-GEL-HF-HK grafts produces specific staining (Fig. 4, C) of human epidermis that allows identification of the wound margin where murine and human epidermis meet.

Results of scoring of HLA-ABC staining of treatments E to H are presented in Fig. 5. C-GAG-HK-HF skin substitutes in which HK had been exposed to FBS (treatment E) were accepted at 33.3% (3 of 9 grafts). Acceptance of C-GAG-HF-HK grafts (treatment G)



Fig. 6. Transmission electron micrographs of healed wounds after treatment with cultured skin substitutes. A, Dermal-epidermal junction of healed skin six weeks after treatment with C-GAG, HF, HK skin substitute. Complete dermal-epidermal junction including anchoring fibrils (AF), lamina densa (LD), and hemidesmosomes (HD) is continuous between the regenerated epithelium composed of HK with intracellular tono-filaments (tf) and connective tissue showing a human fibroblast process (PHF) and banded collagen (C). B, Pigmented spots of skin healed with C-GEL, HF, HK cultured skin shows HK and a melanocyte (HM) with numerous melanosomes (ms) in both types of cells indicative of pigment transfer from HM to HK. Human melanocyte processes (hmp) are identified by their lack of keratin tonofilaments. C, Neuron terminal (NT) in direct apposition to complete dermal-epidermal junction of skin regenerated from C-GAG, HF, HK graft. D, Myelinated neuron (MN) in the perineurial sheath (P) 90 μ m from the dermal-epidermal junction of skin healed with a C-GAG, HF, HK graft. Adjacent are unmyelinated nerves (*). Scale bars = 1 μ m.

improved dramatically to 70% (7 of 10 grafts) by not exposing HK to FBS and by preincubating the material for 24 hours in biochemically defined medium containing 100 ng/ml bFGF and not containing epidermal growth factor or bovine pituitary extract. Similar results were obtained with C-GEL-HF-HK grafts (treatment F) with scoring of 71% (5 of 7 grafts) acceptance. Chisquared analysis revealed that cultured grafts after serum exposure (treatment E) were different (p < 0.01) from xenograft controls (treatment H); no other differences were found.

Transmission electron microscopy. Skin substitutes made from cultured human cells, biopolymers, and growth factors can reorganize to functional analogues of skin tissue. Fig. 6 shows ultrastructural features of closed wounds 6 weeks after treatment with composite skin substitutes, treatments F and G. The dermal-epidermal junction after treatment with C-GAG-HF-HK (treatment G) shows (Fig. 6, A) the presence of keratin tonofilaments attached to hemi-desmosomes, lamina densa and lamina lucida of the basement membrane, and anchoring fibrils extending from the lamina densa into the connective tissue of the repaired skin. Presence of these ultrastructures accounts for the apparently normal strength of the closed wound and for its resistance to damage by lateral or perpendicular forces of moderate magnitude as encountered during routine care and bandage changes during the observation period. However, no quantitative measurements of tensile or shear strengths were made in this study.

Pigmentation of repaired skin is shown by the presence of a human melanocyte in skin repaired with a C-GEL-HF-HK skin substitute (Fig. 6, B). Melanosomes are numerous in the melanocyte, in adjacent transverse profiles of human melanocyte processes, and are also seen at high frequency in the cytoplasms of nearby HK. The presence of melanosomes inside keratinocytes shows the normal process of pigment transfer between melanocytes and keratinocytes after wound closure.

Biopsies from wounds closed with C-GAG-HF-HK

also showed reinnervation of healed skin as shown in Figs. 6, C and 6, D. A nerve terminal was found in direct apposition to the dermal-epidermal junction in healed skin (Fig. 6, C). At approximately the mid-point between the dermal-epidermal junction and the panniculus carnosus, a myelinated nerve and pericyte were seen (Fig. 6, D). The reinnervation of skin healed after treatment with cultured skin substitutes is considered important to the recovery of sensory function, but no examination of sensation was performed in this study.

DISCUSSION

The data in this report show that cultured cells, biopolymers, and peptide growth factors can be transplanted to accomplish wound closure. Although cultured substitutes of human skin consisting of HK only^{27, 28} or C-GEL-HF-HK composites^{17, 31} have been shown clinically to close full-thickness skin wounds, no quantitative studies of wound contraction or graft acceptance for these materials have been reported previously. To directly study the responses of human skin substitutes in a controlled experimental design, the athymic mouse was selected as the host for grafting of the cultured skin substitutes.^{22, 23, 25, 37} Differences between skin wound contraction in the mouse and the human were recognized; and therefore the experimental design required the inclusion of human split-thickness skin (xenograft) as an internal control. For this reason, quantitative comparisons both of wound contraction and of graft acceptance were made among human cultured skin substitutes versus human split-thickness skin (conspecific positive control) and versus no graft (negative control) on this xenogeneic host. The importance of human split-thickness skin as a positive control in the experimental design is reflected by the smaller mean area of human split-thickness skin compared to the murine autograft after 6 weeks of healing.

Epidermal proliferation and differentiation. To promote tissue regeneration after injury, cells must repopulate the wound, proliferate, and differentiate to restore tissue anatomy and physiology. Culture of non-transformed human cells results in logarithmic increases of cell numbers.¹⁹⁻²⁰ Although differentiated epithelium in cultured skin provides some mechanical protection during the period immediately after surgery, proliferative keratinocytes in cultured grafts provide the source of corneocytes that form the protective stratum corneum of healed epidermal skin. Because proliferative keratinocytes retain the capability to differentiated, but differentiated keratinocytes become irreversibly growth arrested, ⁴¹ cellular proliferation should be emphasized during preparation of tissue substitutes in vitro.

Biopolymer implants. Implantable biopolymer substrates^{3, 4} serve multiple purposes. Substrates may be optimized biochemically^{42, 43} and may provide a physical vehicle for delivery of cells for surgical transplantation.²⁶ Prior attachment of cultured cells to collagenous substrates, shown in two examples here, eliminates exposure to proteolytic enzymes (i.e., dispase) used for keratinocyte sheets7, 27, 28 immediately before surgery. Histologic examination of implanted biopolymer matrices shows that implant degradation occurs predominantly by phagocytic activity of macrophages. Therefore metabolic clearance of the biopolymer implant is associated with inflammation that can contribute to collagen deposition by fibroblasts and lead to scar formation. Although wounds in athymic mice do not scar to a great degree, it may be desirable to limit the duration of the inflammatory reaction in human wounds after treatment with implanted polymers to avoid scar formation. According to this speculation, lesser amounts of implanted biopolymers would result in less scarring by reducing the amount of material required to be removed from the implant site.

Dermal substitutes of cultured skin separate the epithelial component from the wound bed and must vascularize to allow survival of the cultured epithelium on the surface. Vascularization of dermal substitutes occurs entirely de novo, whereas vascularization of split-thickness grafts occurs by anastomosis of vessels from the wound to vessels in the graft. This mechanistic difference in vascularization between split-thickness grafts and composite cultured grafts requires that dermal substitutes of cultured grafts be sufficiently thin and that vascularization is sufficiently rapid for the epithelium of cultured dermal-epidermal grafts to survive transplantation.

Growth factors and serum. Growth factors are produced by and act on cells to regulate specific cellular responses that define, in part, the proliferative and differentiated functions of native and regenerative tissues. Epidermal growth factor⁴⁴ is widely recognized to stimulate mitosis in vitro of cultured fibroblasts^{10, 11} and keratinocytes^{14, 45, 46} to promote rapid expansion of cell numbers. More recently, bFGF has been shown to stimulate not only fibroblast growth,32 but also to replace epidermal growth factor as the principal mitogen for HKs grown in biochemically defined medium.³⁴⁻³⁶ In addition, bFGF is known to be a potent mediator of angiogenesis,³³ which is essential for permanent acceptance of cultured grafts onto wounds. The results of this study support the hypothesis that bFGF contributes to increased graft survival by stimulation of epithelialization by cultured keratinocytes and of vas-

cularization from the wound bed to improve graft survival during transplantation (treatment G). Conversely, data for wound contraction (treatments B to E) and HLA-ABC staining (treatment E) also support the recognized fact that exposure of cultured keratinocytes to FBS in vitro causes growth arrest and induces epidermal differentiation.^{3, 45, 47} The exposure of keratinocytes to serum is associated with poor graft acceptance and wound contraction that is not statistically different from no graft controls. Exposure of skin substitutes to bFGF after serum (treatments C and D) did not improve these parameters of wound closure. Therefore poor graft acceptance and pronounced contraction may result, in part, from irreversible growth arrest of cultured keratinocytes by factors in serum, among which is transforming growth factor- β .⁴⁵ Although nonnative stimulators of cyclic adenosine monophosphate^{48, 49} (i.e., choleratoxin) can overcome keratinocyte growth arrest by serum, xenogeneic serum proteins have also been shown to stimulate an antibody response in patients receiving keratinocyte grafts that were grown in medium containing FBS.⁵⁰ Serum-free (with 0.5% vol/vol bovine pituitary extract) or biochemically defined culture conditions (without tissue or blood by-products) after addition of keratinocytes to cultured skin grafts allow depletion of xenogeneic proteins, elimination of serum factors that cause keratinocyte growth arrest, and the administration of pharmacologically active compounds (growth factors, antimicrobials⁵¹, antiinflammatory drugs) to modulate wound healing. This model represents an initial step toward development of a pharmaceutically pure culture medium for transplantation of human somatic cells.

Skin regeneration. Anatomic restoration of skin in repaired wounds must precede restoration of function. Cultured skin substitutes restore the essential functions of skin, protection from microbial infection and control of fluid loss. However, clinical reports regarding pigmentation and innervation of repaired skin are few. In this study, focal pigmentation occurred in cultured skin grafts in which the keratinocyte populations were not exposed to serum. Ultrastructural analysis of the pigmented areas verified that increase in size of the pigmented spots corresponded to transfer of pigment from melanocytes to surrounding keratinocytes. Restoration of normal pigmentation of grafts may add protection from solar radiation to the functions of cultured skin. Regeneration of myelinated axons was also observed in repaired dermis of skin substitutes made from C-GAG (treatment G). Observation of nerve terminals adjacent to the dermal-epidermal junction in

skin healed after treatment with cultured skin substitutes is unprecedented. However, the sensitivity of perception in healed skin has not yet been studied. Also unique to this study is the formation of complete basement membrane including anchoring fibrils within 42 days, as compared with periods of 90 days or more reported with the use of enzymatically released sheets of cultured keratinocytes.⁵²⁻⁵⁴ This difference is attributed predominantly to formation in vitro of biologic attachments between cultured cells and biopolymer substrates several days before transplantation and to the elimination of enzymatic treatment of cultured grafts immediately before surgery. Epidermal adnexa (hair follicles, sweat and sebaceous glands) and other dermal substructures, such as piloerector muscles develop during fetal gestation⁵⁵ and are not regenerated from cultured skin or split-thickness skin during wound healing. Because of their obvious anatomic and physiologic differences from native skin, skin substitutes made from cultured cells, biopolymers, and growth factors are misrepresented by terms such as "skin equivalent."^{4, 5, 17, 31}

Prospective applications for skin substitutes made from cultured human cells, biopolymers, and growth factors include improved therapies for burns, plastic and reconstructive surgery, and dermatologic surgery; alternatives to animal use for in vitro toxicology; and models of skin for study of skin biology and pathology. For surgical applications, the major advantage of cultured skin substitutes is reduction of the donor site of the skin graft, which can allow reduced numbers of operations and shorter hospitalization time after major injuries that require skin grafting. The findings of this report offer additional evidence to suggest that cultured skin substitutes may contribute to reduction of death and morbidity from major skin loss injuries.

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