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Methods for the Serum-Free Culture of Keratinocytes and Transplantation of Collagen–GAG-Based Skin Substitutes

Steven T. Boyce

1. Introduction

Objectives for dermal-epidermal skin substitutes for treatment of acute and chronic wounds include, but are not limited to: increased availability; stimulation of wound healing by transplantation of parenchymal cells; regulation of wound healing responses; and, predictable composition and efficacy to reduce mortality and morbidity. Particularly, the importance of including a cellular component has been demonstrated in experimental grafts of cells and biopolymers *(1–7)*. Transplanted cells may include normal, nontransformed populations isolated for primary culture, or genetically modified cells to deliver specific gene products of therapeutic interest *(8–10)*. Nontransformed cells may include autologous, allogeneic, or chimeric populations within composite grafts. A variety of approaches are directed toward repair of skin wounds by restoration of the functional anatomy and physiology of skin.

An hypothesis shared by several laboratories presumes that duplication of native anatomy and physiology of skin will provide a level of efficacy comparable to split-thickness skin graft. This common rationale has generated models that differ mostly in their selection of biopolymer for delivery of skin cells. Acellular dermis has been populated with keratinocytes and fibroblasts in preclinical models *(11,12)*. Biosynthetic analogs of skin have combined cultured skin cells with polylactic/polyglycolic (PLA/PGA) fabric *(13,14)*, polyethylene oxide/polybutylene terephthalate (PEO/PBT) *(3,15)*, collagen gels *(16–18)*, and collagen–glycosaminoglycan (GAG) sponges *(7,19,20)*. This chapter describes specific techniques for preparation and grafting to surgical wounds in athymic mice of cultured skin substitutes (CSS) from collagen–GAG substrates

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populated with normal human keratinocytes, melanocytes, and fibroblasts grown in serum-free or low-serum conditions.

Principles for CSS based on collagen–GAG substrates that duplicate native skin include separation of epidermal and dermal cells into respective histologic compartments, reformation of epidermal barrier and basement membrane before grafting, regeneration of skin pigmentation, and restoration of a vascular plexus. CSS before grafting possess these properties, except for cellular components of the vasculature. However, addition of endothelial cells to analogs of cornea *(6)* has been reported to stimulate more rapid and complete morphogenesis of the corneal epithelium. Conversely, absence of vascular cells and precursors confers limitations to clinical efficacy of CSS, including increased time to healing, and graft loss from microbial contamination and nutrient deprivation *(21–24)*. Furthermore, any functional phenotypes of CSS that develop in vitro are not stable, and eventually deteriorate unless they are transplanted and engraft to a viable wound.

Anatomic and physiologic deficiencies of CSS impose additional considerations to their clinical use. Therefore, clinical protocols to compensate for deficiencies of CSS must be designed to optimize their efficacy. Conformity with local protocols for surgical and nursing standards may expedite compliance of the staff with modalities of wound care. With the model of CSS described here, its surgical application in a single procedure permits its administration according to prevailing standards of burn treatment *(25–27)*. These standards have greatly influenced the development and modification of preclinical protocols described below. But, ultimately, experimental CSS must provide comparable efficacy to the prevailing standards of wound care, which depend on objective assessment of outcome *(28–31)*. After demonstration of efficacy, factors of cost-effectiveness must also be considered *(32)*. If all these requirements are satisfied, then reduction of mortality and morbidity with CSS can be realized. In the following section, materials and methods are described for culture of human skin cells, preparation of CSS from cells and collagen–GAG substrates, and closure of surgical wounds in athymic mice for preclinical studies with CSS.

2. Materials

Below are summarized the solutions and other materials used for isolation and culture of human epidermal keratinocytes and melanocytes, and dermal fibroblasts; preparation of CSS; and grafting of CSS to athymic mice. These materials provide consistent preparation and engraftment of CSS. Unless stated otherwise, all organic reagents were obtained from Sigma (St. Louis, MO); and inorganic reagents were obtained from Fisher Chemical (Fairlawn, NJ).

2.1. Solutions

1. Isotonic HEPES buffered saline (HBS).
2. 5% v/v Dettol (Reckitt and Colemen, Hull, UK).
3. 2.4 U/mL Dispase II (Boehringer Mannheim, Indianapolis, IN) in HBS.
4. 625 U/mL collagenase (Worthington Biochemicals, Freehold, NJ) in MCDB 153 to activate the enzyme with calcium and magnesium ions, plus 5% v/v bovine pituitary extract (BPE) to neutralize trypsin activities.
5. 0.025% trypsin–0.01% ethylenediaminetetraacetic acid (EDTA) w/v.
6. 10% v/v fetal bovine serum (FBS, Gibco-BRL; Grand Island, NY) in keratinocyte growth medium to neutralize trypsin.
7. Keratinocyte growth medium.
8. Fibroblast growth medium.
9. Melanocyte growth medium.
10. CSS maturation medium, as described below.
11. Basal medium for culture of human epidermal keratinocytes and melanocytes is MCDB 153 *(33–35)*, plus increased concentrations of six amino acids (histidine, isoleucine, methionine, phenylalanine, tryptophan, tyrosine), as described by Pittelkow *(23)*. Human fibroblasts are propagated in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL). Formulations of these media are presented in **Table 1**. These basal media are supplemented as described below to support selective cultures of each respective cell type.
12. Media supplements for selective cultures of skins cells: Supplements to basal nutrient media are added to stimulate mitosis of respective cells. By understanding the mitogenic responses of keratinocytes and melanocytes, basal medium MCDB 153 can be supplemented differently to prepare selective cultures of each cell type. Colony-forming efficiency of cultured keratinocytes in serum-free or biochemically defined medium may be increased by addition of lethally irradiated 3T3 feeder cells *(36,37)*, if strictly defined conditions are not required. DMEM is supplemented with 5% FBS, 10 ng/mL epidermal growth factor, 5 µg/mL insulin, and 0.5 µg/mL hydrocortisone. Media supplements for selective growth of these three cell types are summarized in **Table 2**.
13. HEPES buffered saline (HBS): HBS is formulated as a 10-fold concentrated stock, filter-sterilized, and stored refrigerated at 4°C. The concentrated stock is prepared from 71.49 g HEPES buffer (Research Organics; Cleveland, OH), 10 mL phenol red Stock 5 for MCDB 153 (*see* **Table 1**); 18.02 g glucose, 2.236 g potassium chloride, 76.97 g sodium chloride, and 2.68 g disodium phosphate heptahydrate. Adjust pH of the concentrate to 7.4 before filtration. HBS working solution is prepared by diluting exactly 100 mL of the concentrated stock to 1 L, adjusting the pH to 7.4, and filtering aseptically into a sterile bottle *(34)*.
14. Media for cryopreservation of selective cell cultures: Selective cultures of keratinocytes, melanocytes, or fibroblasts are cryopreserved in 70% v/v of their respective supplemented media, 20% v/v FBS, and 10% v/v dimethyl sulfoxide (DMSO).
15. Collagen–GAG substrates: Collagen–GAG substrates for transplantation of cultured cells are prepared, as described elsewhere *(20)*, to generate a thin (<0.5

Table 1
Basal Media Composition [M/L]

	MCDB 153	DME
Amino acids		
Alanine	1.0e–04	
Arginine	1.0e–03	4.0e–04
Asparagine	1.0e–04	
Aspartic acid	3.0e–05	
Cystine	2.0e–04	
Cysteine	2.40e–04	
Glutamic acid	1.0e–04	
Glutamine	6.0e–03	4.0e–03
Glycine	1.0e–04	4.0e–04
Histidine[a]	2.5e–04	2.0e–04
Isoleucine[a]	7.80e–04	8.0e–04
Leucine	5.0e–04	8.0e–04
Lysine	1.0e–04	8.0e–04
Methionine[a]	9.2e–05	2.0e–04
Phenylalanine[a]	9.2e–05	4.0e–04
Proline	3.0e–04	
Serine	6.0e–04	4.0e–04
Threonine	1.0e–04	8.0e–04
Tryptophan[a]	4.6e–05	8.0e–05
Tyrosine[a]	7.7e–05	5.0e–04
Valine	3.0e–04	8.0e–04
Vitamins and coenzymes		
Biotin	6.0e–08	
Folic acid	1.8e–06	9.1e–06
Lipoic acid	1.0e–06	
Niacinamide	3.0e–07	3.3e–05
D-calcium pantothenate	1.0e–06	8.4e–06
Pyridoxine	3.0e–07	
Pyridoxal	2.0e–05	
Riboflavin	1.0e–07	1.1e–06
Thiamine	1.0e–06	1.2e–05
Vitamin B12	3.0e–07	
Organic compounds		
Adenine	1.8e–04	
Choline chloride	1.0e–04	2.9e–05
Glucose	6.0e–03	5.6e–03
Inositol	4.0e–05	
Myoinositol	1.0e–04	

(continued)

Table 1
Basal Media Composition [M/L]

	MCDB 153	DME
Putrescine	1.0e–06	
Sodium acetate	3.7e–03	
Sodium pyruvate	5.0e–04	1.0e–03
Thymidine	3.0e–06	
Bulk Ions		
Calcium chloride	3.0e–05	1.8e–03
Potassium chloride	1.5e–03	5.3e–03
Magnesium chloride	6.0e–04	
Magnesium sulfate	8.2e–04	
Sodium chloride	1.3e–01	1.1e–01
Sodium phosphate (dibasic)	2.0e–03	
Sodium phosphate (monob)	9.1e–04	
Trace elements		
Cupric sulfate	1.10e–08	
Ferrous nitrate	2.5e–07	
Ferrous sulfate	5.0e–06	
Manganese sulfate	1.0e–09	
Molybdenum	1.0e–09	
Nickel chloride	5.0e–10	
Selenium	3.0e–08	
Silicon	5.0e–07	
Tin chloride	5.0e–10	
Vanadium	5.0e–09	
Zinc sulfate	5.0e–07	
Buffers and indicators		
HEPES	2.8e–02	
NaOH	1.0e–02	
$NaHCO_3$	1.4e–02	2.2e–02
Phenol red	3.3e–06	3.8e–05

[a]Concentrations of six amino acids are increased as described by Pittelkow and Scott ***(23)***.

mm), symmetric sponge that is laminated on one side with a microporous film of the same biopolymer mixture. Dry, acellular substrates are packaged, sterilized by exposure to 2.5 mega-Rad gamma irradiation, and stored at room temperature until they are populated with cultured cells.

Frames to contain cell suspensions over the surface of collagen–GAG substrates after inoculation are cut from type-316 stainless steel to fit 150-mm diameter Petri dishes. Inoculation frames are square and measure exactly 97 mm o.d., and 85 mm i.d. The bottom surface of the frame is beveled from the outside edge

Table 2
Supplements for Selective Culture of Cells from Human Skin

	Keratinocytes	Melanocytes	Fibroblasts
Basal medium	MCDB 153[a]	MCDB 153[a]	DMEM
Epidermal growth factor	1 ng/mL	0	10 ng/mL
Insulin	5.0 μg/mL	5.0 μg/mL	5.0 μg/mL
Hydrocortisone	0.5 μg/mL	0	0.5 μg/mL
Bovine pituitary extract	0.5% v/v	0	0
Fetal bovine serum	0	4% v/v	5% v/v
Penicillin-streptomycin-fungizone	1% v/v	1% v/v	1% v/v
Transferrin	0	0.5 μg/mL	0
Basic fibroblast growth factor	0	0.6 ng/mL	0
α-Melanocyte stimulating hormone	0	17 ng/mL	0
Endothelin-1	0	2.5 ng/mL	0
Vitamin E (α-tocopherol)	0	1.0 μg/mL	0

[a]Plus increased concentrations of hydrophobic amino acids as described in **Table 1**.

to the inside edge, to generate a blunt wedge at the bottom of the frame to increase weight distribution where the frame contacts the substrate, and to decrease leakage of cells. Inoculation frames used in the author's laboratory were fabricated by custom order in a local machine shop. Frames are sterilized by steam autoclaving.

16. Maturation medium for cultured skin substitutes: To stimulate development of epithelial barrier in skin substitutes before grafting, it was observed that the keratinocyte growth medium was not satisfactory. Poor epithelial organization was observed for at least two major reasons: absence of essential fatty acids that are required for barrier formation, and an insufficient calcium concentration. Replacement of calcium alone was not sufficient to permit formation of epidermal barrier in vitro. However, studies to supplement MCDB 153 with lipids *(38)* stimulated partial formation of barrier lipids and structures analogous to stratum corneum of epidermis. This maturation medium is formulated from lipid prestocks (oleic acid, palmitic acid, arachidonic acid; vitamin E) carried in the medium with BSA, carnitine, and increased serine. Specifications of this formulation are summarized in **Table 3**.

Supports to lift skin substitutes to the air–liquid interface are used to fit a 150-mm diameter Petri dish, and consist of three parts. Square, seamless frames measuring 97 mm od and 87 mm id, and circular wire mesh measuring 12.5 cm in diameter, both consist of type-316 stainless steel. Both of these devices were fabricated in a local machine shop. The wire mesh and lifting frames are sterilized by steam autoclaving. The mesh is overlaid with a cotton filter pad (Schleicher and Schuell;

Table 3
Formulation of Lipid-Supplemented Maturation Medium for Skin Substitutes

	Pre-stock		50X Stock		Final conc.
Compound	mg/mL	[M]	µL/mL	[M]	[M]
Linoleic acid	21.05	7.5×10^{-2}	10	7.5×10^{-4}	1.5×10^{-5}
Palmitic acid	32.05	1.25×10^{-1}	10	1.25×10^{-3}	2.5×10^{-5}
Oleic acid	35.30	1.25×10^{-1}	10	1.25×10^{-3}	2.5×10^{-5}
Archidonic acid	10.65	3.5×10^{-2}	10	3.5×10^{-4}	7.0×10^{-6}
α-Tocopherol-Ac	47.40	1.1×10^{-1}	10	1.1×10^{-3}	2.2×10^{-5}
BSA (FFA free)	27.60	4.2×10^{-4}	950	4.0×10^{-4}	8.0×10^{-6}
	Pre-stock		100X Stock		Final conc.
Compound	mg/mL	[M]	µL/mL	[M]	[M]
Serine	105	$1 \times 10^{-9} M$	10	1.0	1.0×10^{-2}
Carnitine	0.161	$1 \times 10^{-9} M$	10	1.0×10^{-3}	1.0×10^{-5}

Keene, NH) that is cut to a 12-cm diameter, and acts as a wick for uniform distribution of medium under air-exposed skin substitutes.

17. Culture vessels: Selective cell cultures of human keratinocytes or melanocytes are grown in 75-cm^2 polystyrene flasks (Corning, Corning, NY). Fibroblasts are grown in 150 cm^2 flasks (Corning). Cultured skin substitutes are inoculated with cells and incubated in 150-mm diameter tissue-culture Petri dishes (Lux-Nunc, Gaithersburg, MD). All cells are cryopreserved in 2-mL screw-capped cryogenic vials (Corning). Assorted sterile tubes and pipets are from Corning, or Falcon (Fisher Scientific, Itasca, IL).
18. Solution for irrigation of wounds grafted with cultured skin substitutes: Solutions for topical irrigation of grafted wounds consisting of nutrients, mitogens, and antimicrobials have been shown to improve engraftment of avascular skin substitutes ***(39,40)***. For preclinical studies the irrigation solution consists of:
    a. Basal nutrient medium MCDB 153 (*see* **Table 1**), supplemented with.
    b. Insulin at 5 µg/mL.
    c. Hydrocortisone at 0.5 µg/mL.
    d. Ciprofloxacin (Cipro™, Miles, West Haven, CT) at 20 µg/mL.
    e. Nystatin at 100 U/mL (Gibco-BRL).

2.2. Animal Surgery

1. Anesthesia: Animals are anesthetized with Avertin, which consists of 25 mg/mL tribromoethanol dissolved in 1.25% tertiary-amyl alcohol ***(41)***. Although this compound provides stable anesthesia for the 30–60 min surgical procedure, any anesthetic with pharmacologic actions appropriate to the procedure may be substituted.

2. Resusitation: Resusitation solution after surgery consists of 3 mg/mL cephtazidime in saline.
3. Surgical supplies: Sterile supplies of several varieties are used to graft skin substitutes to wounds in athymic mice, including:
   a. Betadine™ swabs (Purdue Frederick Company; Norwalk, CT).
   b. 70% isopropyl alcohol swabs (Kendall Healthcare, Mansfield, MA).
   c. Skin Skribe™ surgical markers (Hospital Marketing Services, Naugatuck, CT).
   d. 4 × 4 in., 16-ply cotton gauze sponges (Johnson & Johnson Medical, Arlington, TX).
   e. Ethilon™ 6-0 sutures (Ethicon, Sommerville, NJ).
   f. N-Terface™ (Winfield Laboratories, Dallas, TX).
   g. Xeroform™ gauze (Sherwood Medical, St. Louis, MO).
   h. Vaseline™ gauze (Sherwood Medical).
   i. Steri-Strip™ compound benzoin tincture ampules (3M Surgical Medical, St. Paul, MN).
   j. OpSite™ (Smith & Nephew Medical, Hull, UK).
   k. Coban™ bandage (3M, Surgical Medical, St. Paul, MN).
4. Instruments for surgery:
   a. Dumont™ fine forceps, #3C pointed-tipped, and #2A flat-tipped (Ted Pella, Redding, CA).
   b. Iris scissors, needle driver, bandage scissors, and mosquito hemostat (Miltex Instrument, Lake Success, NY).
5. Aseptic care and housing of athymic mice:
   a. Autoclavable, filter-topped cages, bedding, and chow.
   b. Water containing 0.2 mg/mL benedryl (as diphenhydramine, Elkins-Sinn, Cherry Hill, NJ), and 1% v/v Septra™ (as trimethoprim and sulfamethoxizole, Burroughs-Wellcome, Research Triangle Park, NC).
   c. Heating pads (non-sterile).
   d. Laminar flow cage isolator (Forma Scientific, Marietta, OH).

3. Methods

3.1. Individual Cell Cultures in Selective Media

3.1.1. Primary Cultures of Skin Cells

Keratinocytes, melanocytes, and fibroblasts may be isolated from a single skin biopsy by serial disaggregation using enzymatic and mechanical techniques. Split-thickness skin is preferable to full-thickness skin, because penetration of enzymes is more rapid. All procedures are performed aseptically, and all materials are sterile. Dissecting instruments may be repeatedly sterilized during the procedures by dipping in 95% ethanol, followed by burning the ethanol in a gas flame and cooling the instruments before contacting the tissue.

1. Wash tissue in HBS, and trim away any subcutaneous tissue.
2. Disinfect skin by submersion for 15–30 s in 5% v/v Dettol solution, followed by three washes in HBS.

3. Cut skin into strips 2–3 mm wide, and transfer to Petri dishes containing several layers of cotton gauze saturated with 2.4 U/mL Dispase II in HBS. Incubate skin dermis side against gauze in Dispase II for 30–120 min, until epidermis readily separates from the dermis with forceps. Do not allow skin strips to float. After epidermis has loosened from dermis, transfer the skin strips to a fresh Petri dish containing HBS.
4. Mechanically separate epidermis from dermis, and place epidermis into a fresh Petri dish containing HBS. After all the epidermal sheets have been collected, seal the Petri dish with Parafilm™ and incubate for 3 h at 4°C. Because HBS contains no calcium, this incubation depletes calcium and loosens desmosomal junctions between keratinocytes.
5. Mince dermis into fine (0.5–1 mm^2) pieces, and transfer into a 50-mL centifuge tube containing 625 U/mL collagenase in culture medium, plus 5% v/v BPE, equilibrated to 37°C, and 5% CO_2 (collagenase activity is dependent on divalent cations, i.e., calcium and magnesium). Agitate the tissue pieces with a plugged Pasteur pipet and incubate at 37°C and 5% CO_2 for 30–90 min. Agitate the tissue pieces for 1–2 min at 20–30 min intervals during the digestion. After the tissue has digested to minimal fragments, centrifuge the suspension at 250*g* for 5 min at 4°C (excessive digestion will reduce the yield of viable cells). Aspirate the collagenase and resuspend the pellet in selective medium for fibroblasts, as described in **Table 2**. Repeat centrifugation and resuspension, and inoculate dermal cells and fragments into 150-cm^2 flasks containing 10 mL selective medium for fibroblasts, at an approximate ratio of 1 cm^2 dermis:50 cm^2 of culture surface. After 16–24 h, add 15 mL of medium per flask. Refresh medium at 2-d intervals.
6. Retrieve epidermal strips from 4°C incubation and collect HBS from dish. Mince epidermal strips into fine pieces, as was done with dermis. Transfer tissue fragments to a 50-mL centrifuge tube, add 1–2 mL of 0.025% trypsin–0.01% EDTA per cm^2 of epidermal tissue, and agitate 4 min using a plugged Pasteur pipet. Let tissue fragments settle, collect the cell suspension, and transfer it to selective medium for keratinocytes with 10% FBS on ice. Resuspend the epidermal fragments in HBS, agitate for 4 min, collect the cell suspension, and transfer to keratinocyte medium plus 10% FBS. Repeat resuspension, agitation, and collection of cell suspension into keratinocyte medium plus 10% FBS. Resuspend the epidermal fragments in a small volume of keratinocyte medium containing 10% FBS in a separate tube. Centrifuge the suspension (s) and epidermal pieces at 250*g* for 5 min at 4°C, and aspirate the supernatants. Resuspend the epidermal cells and tissue fragments into a small volume of selective medium for keratinocytes. Count the epidermal cell suspension. To grow keratinocytes, inoculate into 75-cm^2 flasks at 2–3 × 10^4 cells/cm^2 containing 15 mL of selective medium for keratinocytes, equilibrated to 37°C and 5% CO_2. To grow melanocytes, inoculate into 75-cm^2 flasks at 1 × 10^5 cells/cm^2 containing 15 mL of selective medium for melanocytes ***(42)*** (*see* **Table 2**), equilibrated to 37°C and 5% CO_2. Refresh keratinocyte medium at 2-d intervals. Refresh melanocyte medium three times per wk.

3.1.2. Cryopreservation of Cells by Controlled Rate Freezing

Before cells become confluent in primary culture, great efficiency is gained if they are cryopreserved for later use. A representative procedure for cryopreservation of keratinocytes is described below, but it may be modified for other cell types by substitution of respective media for cryoprotection.

1. Prepare cryopreservation medium by combining 7 parts selective medium for keratinocytes, plus 2 parts FBS, and 1 part dimethylsulfoxide. Sterilize by filtration through a 0.22-μm filter.
2. Harvest keratinocytes by washing cells with HBS, aspiration of HBS, and addition of 1 mL/75-cm^2 flask of cold 0.025% trypsin–0.01% EDTA. Incubate for 3–4 min at 37°C, or 4–5 min at room temperature. Gently tap the flask against the bench and examine in a phase-contrast contrast microscope. After greater than 90% of the cells have detached, collect the cells by serial washing of multiple flasks with a single 10 mL aliquot of HBS, and transfer the cell suspension to selective medium for keratinocytes, plus 10% FBS on ice. Repeat the HBS wash and combine suspensions. Centrifuge cells at 250*g*, aspirate the supernatant, resuspend in a small volume of selective medium for keratinocytes, and count cells. Repeat centrifugation and aspiration of supernatant, and resuspend keratinocytes in cryopreservation medium for keratinocytes at $0.5–3.0 \times 10^6$ cells/mL. Dispense into cryogenic vials, and freeze at a controlled rate (i.e., 1°C/min) in a CyroMed microprocessor-controlled freezer (Forma Scientific, Marietta, OH) with a program for suspensions of cultured cells, to retain best viability. If a controlled-rate freezer is not available, place vials into a sealed block of styrofoam with at least 1 cm covering all sides of all vials, place into a –70°C freezer overnight, and transfer to liquid nitrogen the next day. Store cells indefinitely in liquid nitrogen.

3.1.3. Recovery of Cryopreserved Cells into Culture

Recover keratinocytes by thawing rapidly in 70% isopropyl alcohol, making sure that no alcohol leaks into the vials as they warm. To assure the seal on the vial, swab the vial with 70% isopropyl alcohol, allow it to dry, release pressure from the vial by loosening and resealing the cap, and place the vial into 70% isopropyl alcohol in a 37°C water bath. As soon as keratinocytes are thawed, inoculate them at $0.3–1.0 \times 10^4$ cells/cm^2 into 75-cm^2 flasks containing 15 mL of selective medium for keratinocytes equilibrated to 37°C and 5% CO_2. Refresh medium after 12–24 h, and at 2-d intervals thereafter. Fibroblasts and melanocytes are handled similarly, but in their respective media. Incubate until sufficient cells are available to prepare cultured skin substitutes. Representative photomicrographs of selective cultures of keratinocytes, melanocytes, and fibroblasts, in log-phase growth after recovery from cryopreservation, are shown in **Fig. 1**.

3.2. Preparation of Cultured Skin Substitutes

A schematic diagram for preparation of cultured skin substitutes is presented in **Fig. 2**. Selective cultures of skin cells are harvested on successive days, and

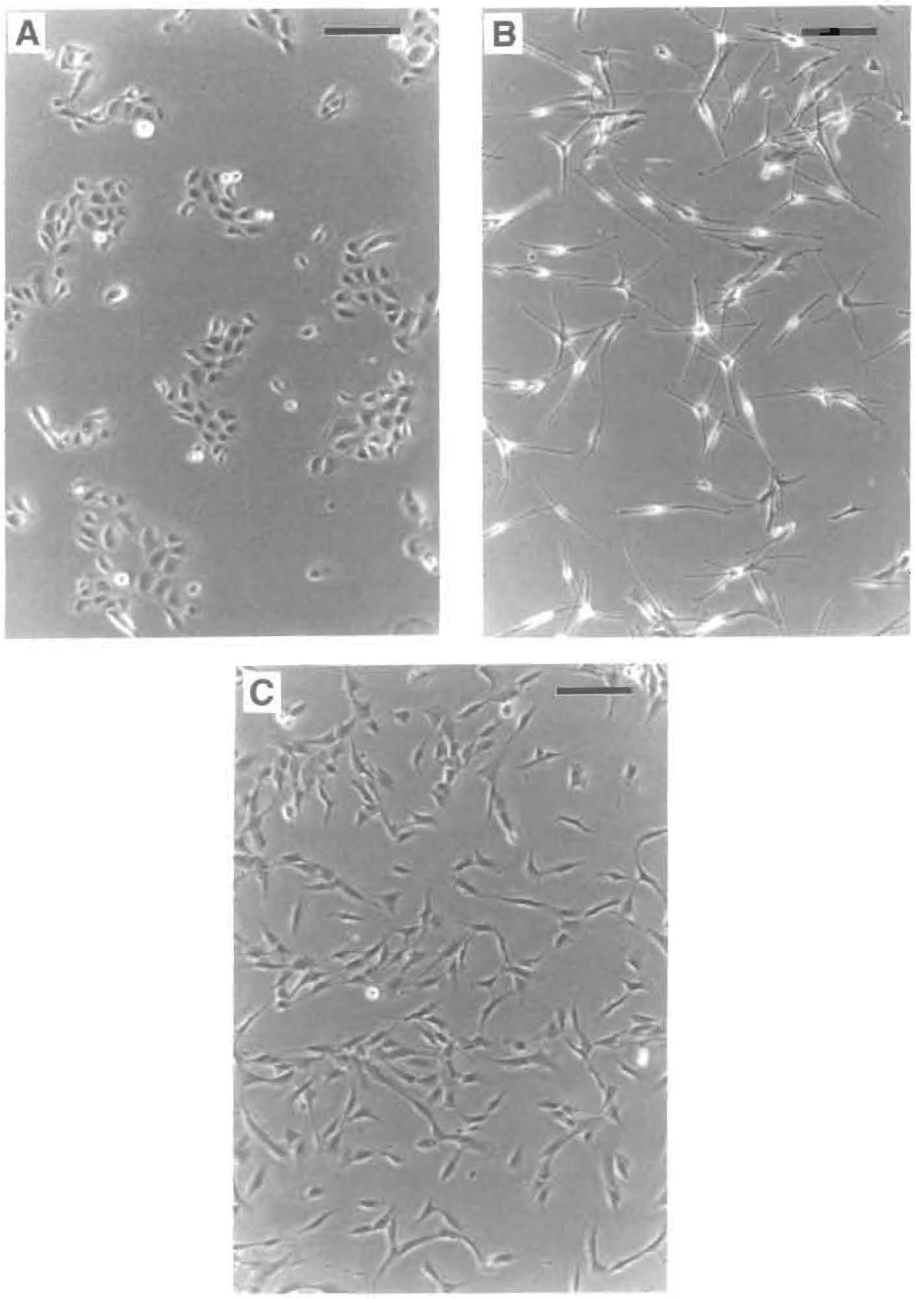

Fig. 1. Photomicrographs of normal human skin cells in log-phase, selective culture. **(A)** epidermal keratinocytes; **(B)** epidermal melanocytes; and, **(C)** dermal fibroblasts. Scale bar, 0.1 mm.

inoculated onto collagen-glycosaminoglycan substrates in submerged culture. Epithelium matures into an analog of epidermis by air-exposed incubation in lipid-supplemented medium. Epidermal melanocytes may be added to the skin substitute by co-inoculation with keratinocytes at a ratio of 1 melanocyte/30 keratinocytes.

3.2.1. Rehydration of Dry Collagen–GAG Substrates

Rehydrate dry collagen–GAG substrates (10 × 10 cm) by removal from sterile packaging, and place into 150 mm tissue-culture Petri dishes containing 75 mL HBS. Incubate 30 min and invert substrate in dish. Aspirate and refresh HBS, gently express air from the substrate, and incubate 30 min. Repeat procedure using selective medium for fibroblasts. Aspirate medium from dish, orient the substrate with porous side up, and install inoculation frame (*see* **Subheading 2.1.5.**) over substrate. Add 20 mL of selective fibroblast medium only to outside of frame, and equilibrate in cell culture incubator at 37°C and 5% CO_2.

3.2.2. Harvest and Inoculation of Cultured Fibroblasts

Harvest fibroblasts in log-phase growth, as described for keratinocytes in **Subheading 3.1.2., step 2**, except reduce the incubation time of fibroblasts in 0.025% trypsin–0.01% EDTA to 2–3 min. Collect cells in 10 mL HBS, transfer to a 50-mL centrifuge tube containing selective medium for fibroblasts, centrifuge at 250*g*, aspirate supernatant medium, resuspend fibroblasts in a small volume of their selective medium, and count cells. Adjust cell density to 4×10^6 cells/mL. Evenly inoculate 10 mL of the fibroblast suspension over the area within the inoculation frame (5×10^5 cells/cm^2). After 24 h, remove the inoculation frame, aspirate medium and unattached cells, and transfer the substrate with fibroblasts to a new 150-mm Petri dish. Wash the substrate with attached fibroblasts in 35 mL selective medium for keratinocytes, and carefully invert the dermal substitute, to orient it with the microporous side up, for inoculation of keratinocytes. Wash dermal substitute with 35 mL of selective medium for keratinocytes, aspirate medium, and install an inoculation frame, as before. Add 20 mL selective medium for keratinocytes only to the outside of the frame, and equilibrate in cell-culture incubator at 37°C and 5% CO_2.

3.2.3. Harvest and Inoculation of Cultured Keratinocytes

Harvest keratinocytes as described in **Subheading 3.1.2., step 2**, and, after centrifugation, resuspend the cell pellet in a small volume of selective medium for keratinocytes. Count the keratinocytes, and adjust the density to 8×10^6 cells/mL. Remove dermal substitute with inoculation frame from the incubator, and evenly inoculate 10 mL of the keratinocyte suspension over the area within the inoculation frame (1×10^6 cells/cm^2). Inoculation of keratinocytes onto dermal substitutes is defined as day 0 of incubation of cultured skin substitutes.

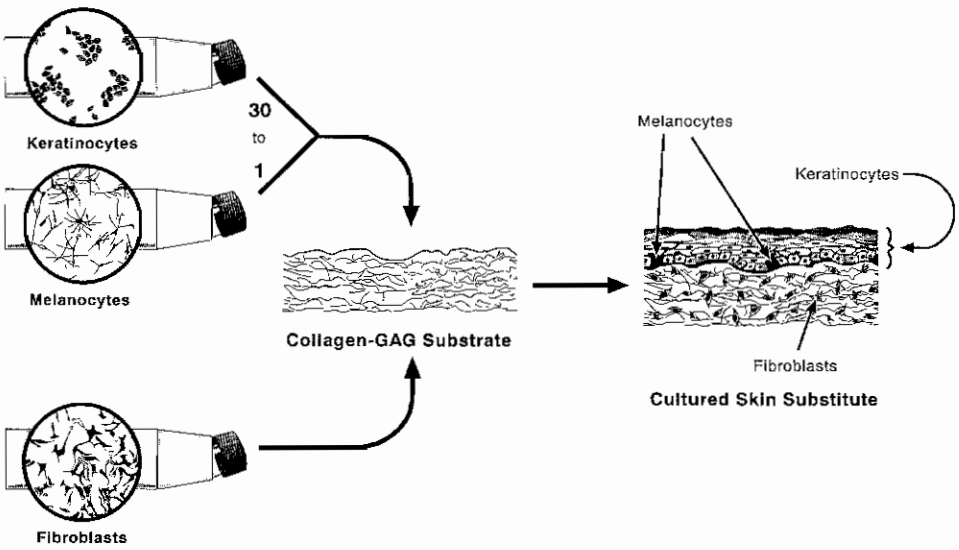

Fig. 2. Schematic diagram of preparation of cultured skin substitutes. Selective cultures of dermal fibroblasts are inoculated into the porous reticulations of a collagen-glycosaminoglycan (GAG) substrate. Selective cultures of epidermal keratinocytes are prepared to include or exclude selective cultures of epidermal melanocytes. If added, melanocytes are mixed with keratinocyte suspensions at a ratio of 1 melanocyte to 30 keratinocytes before inoculation onto the micro porous surface of a collagen–GAG substrate. The cell-biopolymer composite is incubated at the air-liquid interface to promote epithelial stratification and barrier formation.

3.2.4. Addition of Melanocytes to Cultured Skin Substitutes

Add melanocytes to skin substitutes by simultaneous harvest of selective cultures of melanocytes. After each cell type is counted, and before inoculation of keratinocytes, add melanocytes into the keratinocyte suspension at a ratio of 1 melanocyte:30 keratinocytes. Inoculate dermal substitutes, as described in **Subheading 3.2.3**.

3.2.5. Incubation Day 1

After 24 h (incubation d 1), add 10 mL selective medium for keratinocytes to the inside of the inoculation frame only.

3.2.6. Incubation Day 2

On incubation d 2, remove the inoculation frame and wash with maturation medium containing the lipid supplement described in **Table 3**. Increase the calcium concentration in the medium to 0.5 m*M*, as described in **Table 4**, and

discontinue BPE. Incubate the cultured skin substitute submerged in medium.

3.2.7. Incubation Day 3

On incubation d 3, prepare a new 150-mm Petri dish containing a lifting frame, mesh and cotton support (*see* **Subheading 2.1., item 6**). Add 60 mL of maturation medium to the dish according to the formulation and schedule in **Table 4**. Equilibrate the assembly in a cell-culture incubator at 37°C and 5% CO_2. Aspirate medium from the dish containing the skin substitute, and trim away edges of the collagen–GAG substrate that were outside of the inoculation frame (no cells), and discard. Remove the Petri dish with lifting frame from the incubator, and transfer the cultured skin substitute to the top of the cotton pad on the stainless steel mesh. Transfer the skin substitute by laying a piece of N-terface of correct size (8.5 × 8.5 cm) on the epithelial surface. Lift one corner of the skin substitute and N-terface with a flat-tipped forceps, and hold an adjacent corner with another identical forceps. Lift the skin substitute, transfer it to the dish containing the lifting frame, and lay it on the cotton pad saturated with medium. To reposition the skin substitute, lift it from one site and replace it in another site, but do not drag or pull it. Return the lifted skin substitute to the incubator.

3.2.8. Incubation Day 4

On culture d 4, aspirate medium from the dish containing the skin substitute, and add 45 mL maturation medium for d 4, as described in **Table 4**. Incubate the skin substitute in this condition until use. Refresh maturation medium daily.

3.2.9. Preparation of Cultured Skin Substitutes for Surgery

On the day before animal surgery (ca incubation d 14), prepare cultured skin substitutes for grafting. Transfer skin substitutes to new 150-mm Petri dishes containing 10 mL maturation medium. With a straight-edge razor blade (Weck, Weck, NC) held in a hemostat and a fine-tipped forceps, cut skin substitutes into grafts exactly 2 × 2 cm. Transfer the individual grafts to the dish containing the lifting frame, and return to the incubator until the day of surgery. A photomicrograph of the histology of a representative skin substitute is presented in **Fig. 3**.

3.3. Animal Surgery

All care and use of animals is approved by the Institutional Animal Care and Use Committee of the University of Cincinnati. All procedures are performed with aseptic technique, and all materials are sterile. Surgical procedures are performed in a biological safety cabinet, and animals are housed in filter-topped cages in a laminar flow cage isolator.

Table 4
Schedule and Media for Maturation of Cultured Skin Substitutes

Factor	Day 1	Day 2	Day 3	Day 4 and after
BPE (% v/v)	0.5	0	0	0
Lipid supplement[a]	No	Yes	Yes	Yes
EGF (ng/mL)	1	1	1	0
Calcium (m*M*)	0.2	0.5	1.0	1.5
Air-exposure	No	No	Yes	Yes

[a]Lipid Supplement is formulated as described in **Table 3**. All media are MCDB 153 as described in **Table 1** supplemented with 5 μg/mL insulin and 0.5 μg/mL hydrocortisone.

3.3.1. Preparation of a Sterile Field for Surgery

Prepare materials for surgery by packaging of multiple units of each item into sterile specimen jars. Cover the inside of the biological safety cabinet with a sterile drape. Place electric heating pads set on "low" beneath the operating field, and beneath a cage to be used for recovery, to prevent hypothermia during and after surgery.

3.3.2. Anesthesia

Anesthetize an athymic mouse by intraperitoneal injection of 350–500 μL of avertin, as described in **Subheading 2.2.1**. Verify complete anesthesia by negative response to toe pinch.

3.3.3. Cleansing of the Surgical Site

Cleanse skin on (right) flank of animal by serial swabbing with Betadine, followed by 70% isopropanol. Demarcate a 2 × 2-cm area on the flank with a square template by marking the corners and midpoints of the sides of the template.

3.3.4. Preparation of a Full-Thickness Skin Wound

Carefully prepare a full-thickness skin wound to preserve the panniculus carnosus. With fine forceps and iris scissors, lift a corner of the demarcated site and incise through the epidermis and into the dermis. Continue the incision around the entire wound perimeter by placement of the scissors tip into the dermis, application of gentle forward and upward pressure, and closing the scissors to advance the incision. Apply gentle countertraction to the edges of the incision to promote its advancement. Periodically open the incision and verify visually that the panniculus carnosus remains intact. After the incision is complete, carefully lift one corner of the murine skin with a fine forceps, and dissect the panniculus carnosus from the dermis with the tip and edge of the scissors. An example of the completed dissection is presented in **Fig. 4A**.

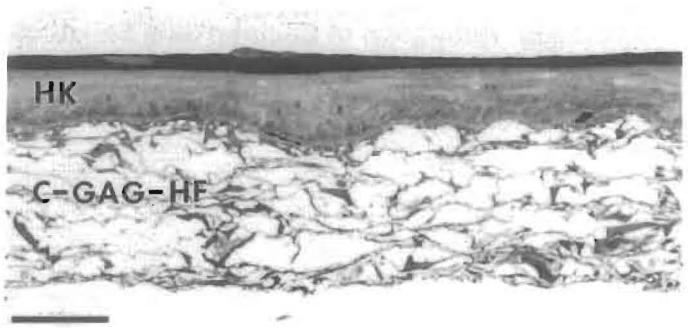

Fig. 3. Histology of a cultured skin substitute. Keratinocytes (HK) are restricted to the outer surface of the dermal substitute (C-GAG-HF) to form separate compartments within the skin substitute. Total thickness is less than 0.5 mm. Scale bar, 0.1 mm.

3.3.5. Suturing and Dressing of Cultured Skin Substitutes onto Wounds

Place the cultured skin substitute with a backing of N-terface onto the wound. Secure the graft and dressing to the wound margin with stent-type suturing at eight points, as shown in **Fig. 4B**. Pack the graft with several layers of cotton gauze, and tie opposing sutures together, as shown in **Fig. 4C**. Apply benzoin adhesive around perimeter of wound, and cover the packing with OpSite to form a compartment over the graft that retains fluid, but is vapor permeable.

3.3.6. Resusitation

Resusitate the animals by intraperitoneal injection of 1.0 cc saline containing 3 mg of cephtazidime.

3.3.7. Bandaging of Dressings

Bandage the dressed graft with Coban, and prepare a small (3–5 mm) opening to inject irrigation solution. Inject 1.5 cc of irrigation solution (*see* **Subheading 2.1.8.**) through OpSite into gauze. Maintain mice on the heating pad until they recover from anesthesia. After animals recover from anesthesia, return them to their respective cages for housing. Keep cages on heating pads set on "low" until all dressings are removed. Supply water prepared with benedryl and Septra as described in **Subheading 2.2.5.2**.

3.4. Dressing and Care Protocol

3.4.1. Postoperative Days 1–14

Inject 1.0 cc/animal/d of irrigation solution into gauze packing over the wound.

3.4.2. Post-operative Day 14

Remove dressings and sutures and collect data (i.e., photographs, area tracings, noninvasive instruments) ***(43,44)***. Replace dressings with N-terface contacting the graft, followed by Xeroform, cotton gauze, and Coban bandage until d 21.

3.4.3. Post-operative Day 21

Remove dressings, collect data and redress with N-Terface, cotton gauze, and Coban until d 28. Representative healing of wounds at d 21 after surgery is shown in **Fig. 5**. Healed skin from skin substitutes depleted of melanocytes is shown in **Fig. 5A**. Confirmation of engraftment of human keratinocytes is shown in **Fig. 5B** by immuno histochemical staining for HLA-ABC antigens ***(2,40,45)***. Skin substitutes with added melanocytes produce uniformly black skin, as represented in **Fig. 5C**. Localization of melanocytes within the basal layer of keratinocytes is verified by immunohistochemical staining for the melanocyte marker, Mel-5 ***(46)***.

3.4.4. Postoperative Day 28

Remove all dressings and swab healed skin with vaseline gauze. Collect data as needed.

4. Notes

4.1. Media Preparation

1. Storage of media: To increase reproducibility, prepare sufficient media and supplements for 2–3 mo, aliquot, and freeze at –20°C or –70°C. This simple practice also decreases labor intensity of medium preparation.
2. Reconstitution and supplementation of media: Reconstitute and supplement media for use not less than weekly. Even serum-supplemented media deteriorate at 4°C. Optimum performance of serum-free media is obtained if all components are as fresh as possible. Serum-free media are more subject to reduced performance if any component (s) lose (s) activity.
3. Use of media:
   a. Warm media to 37°C before use, minimize warm time.
   b. Keep labile reagents (especially trypsin) on ice during use.
   c. Avoid exposure of culture media to light, especially fluorescent light that degrades certain vitamins (i.e., riboflavin, B_2).
   d. Replace unused media chemicals no less than annually.

4.2. Cell Culture

4. Safety: Maintain universal precautions for laboratory staff. Human cells can carry pathogens (i.e., human immunodeficency virus, hepatitis, cyto megalo virus). Certain sources (e.g., surgical discard) of human tissues may not require pathogen testing. Other sources (e.g., tissue banks accredited by the American Association of Tissue Banks) perform tests for human pathogens, and require negative

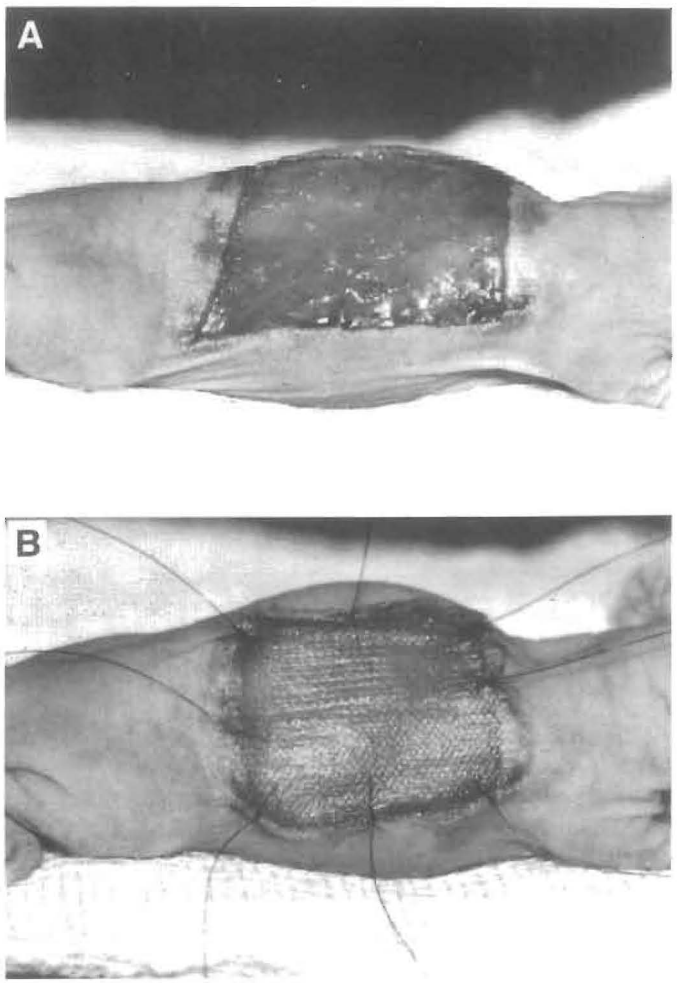

Fig. 4. Photographs of steps in the procedure to graft human cultured skin substitutes to athymic mice. **(A)** Murine skin removed with preservation of the panniculus carnosus. **(B)** Cultured skin substitute covered with N-Terface™ dressing is secured to the wound margin with stent-type sutures. *(continued on opposite page)*

tests before release of tissue. However, negative tests for pathogens do not provide absolute assurance that pathogens are not present. Therefore, appropriate safety factors should be required to protect staff who handle human-derived materials. All human tissues and materials (media, culture vessels) that contact human cells and tissues should be treated as biohazards, and be treated lethally (e.g., bleach, incineration) for disposal.

5. Tissue: Isolate cells from tissue of optimum viability. Discarded tissue from elective surgery (i.e., neonatal circumcision, reduction mammoplasty) may be

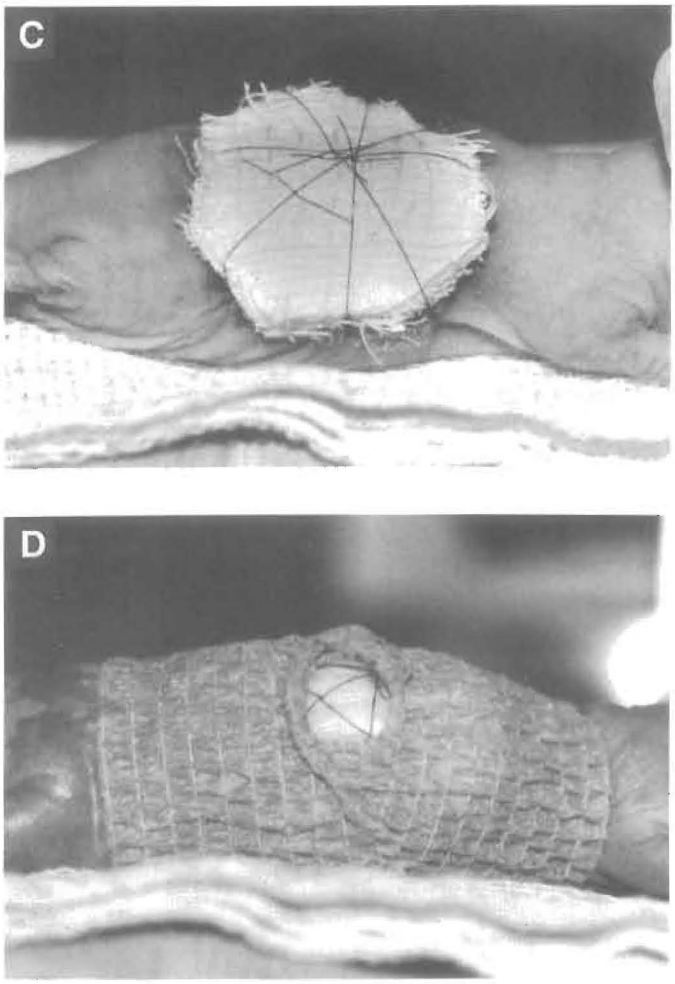

Fig. 4. **(C)** Grafted wound is packed with gauze and stent sutures are tied over to secure the packing. **(D)** Dressed wound is covered with OpSite™ to retain moisture in the wound, and is bandaged with Coban™. Irrigation solutions are injected through a small aperture in the Coban™, through the OpSite™ and into the gauze to modulate the healing wound.

obtained according to guidelines and policies of the local institutional review board. Cadaveric donors who are young (18–30 yr), otherwise healthy individuals are usually sources of high proportions of proliferative cells. Samples of split-thickness skin are best. Minimize ischemic time between acquisition of tissue and isolation of cells to preserve optimum cellular viability.

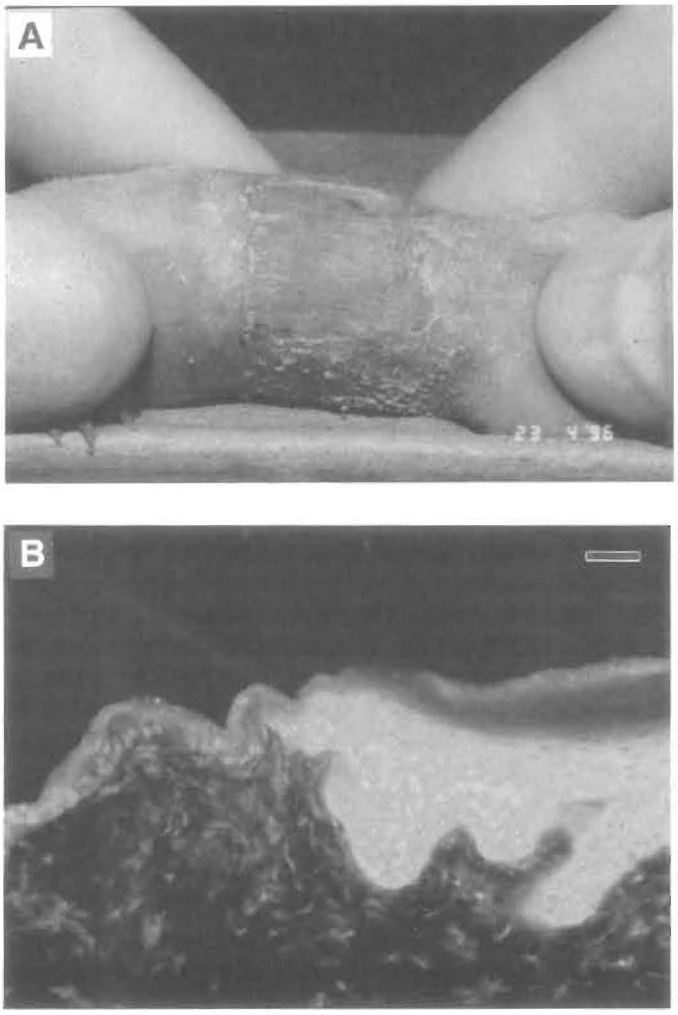

Fig. 5. Photographs of nonpigmented and pigmented human skin after engraftment of cultured skin substitutes. **(A)** By 21 d after grafting, the epithelium is fully functional and dry. **(B)** Persistence of human cells is verified by immuno histochemical staining of HLA-ABC antigens in healed epidermis (right of panel). Staining stops abruptly at the wound margin (center of panel), and murine epidermis (left of panel) is negative for human cell markers. **(C)** Cultured skin substitutes with added melanocytes generate black skin by 21 d after grafting. Localization of melanocytes to the basal layer of keratinocytes (arrowheads) is confirmed by immuno histochemical staining with the melanocyte-specific marker, Mel-5. The outer surface of the stratum corneum is identified with asterisks (*). Scale bar, 0.1 mm.

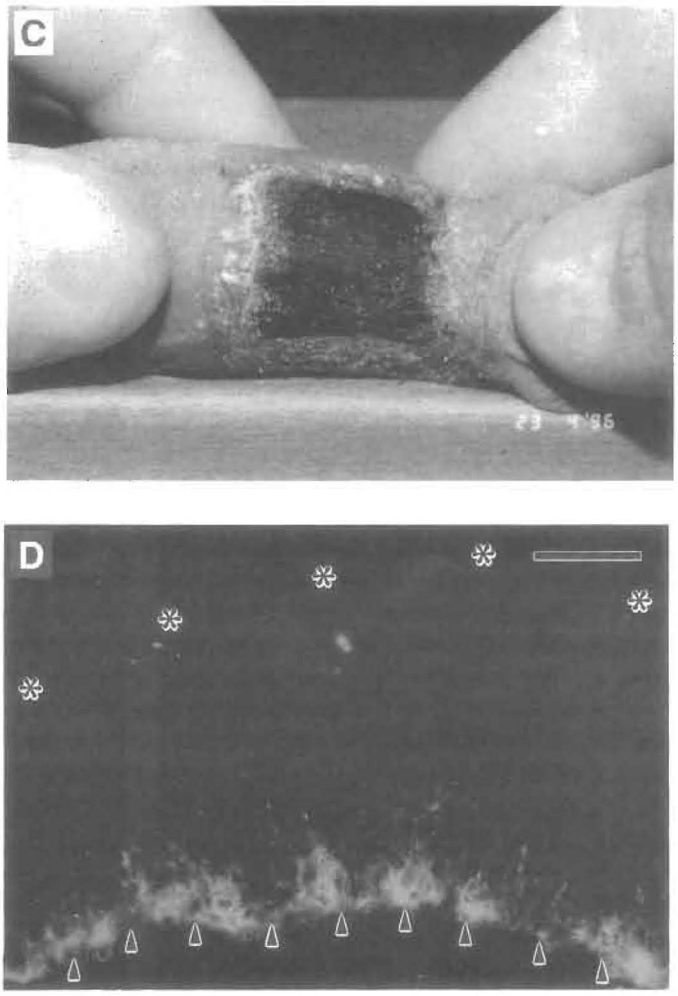

Fig. 5C and D.

6. Cryopreservation: Cryopreserve cells after primary culture in liquid nitrogen for long-term storage. If sufficiently large cell populations are propagated in primary culture, they may be distributed into aliquots and used for months or years. This simple step will assure continuous quality of results, and provide a performance standard for cellular responses to experimental conditions in preclinical studies.
7. Cell viability: Avoid repeated subculture before assay or surgery. All non-transformed cells have finite life spans, and cellular responses decline with time in culture.

4.3. CSS Preparation

8. Inoculate cells from log-phase cultures. Avoid confluence and density inhibition of keratinocyte cultures, especially. Density inhibition is not fully reversible, and, if keratinocytes become arrested, the epithelium of the skin substitute will be compromised.
9. Determine kinetics of optimum cellular viability and differentiation in vitro to predict the best surgical result. If skin substitutes are grafted too early, then stratification and barrier may not be optimal; if too late, then potential for cellular proliferation may be reduced.
10. Handle skin substitutes with a flexible, inert, nonadherent backing (i.e., N-terface polypropylene dressing) to reduce folding and increase mechanical strength during processing in vitro and surgical procedures.

4.4. Animal Surgery

11. Use a single gender of animals to reduce physiologic variations, and gender–gender interactions. Female athymic mice are very tolerant of handling and surgery.
12. Use animals 2–4 mo of age (young adults) to provide greatest vigor after surgery, and an observation period of more than 1 yr. Use animals from the same breeding lot for each experiment to avoid artifacts caused by age or other factors.
13. Be very careful to preserve the integrity and blood flow of the panniculus carnosus to optimize the vascularity of the woundbed for grafts. Although the panniculus carnosus is not required for engraftment of skin substitutes ***(11)***, its presence increases the probability for engraftment.
14. Place chow cakes in the bedding of the cage for first 4 wk after surgery.
15. Check animals no less than daily while in dressings.

4.5. Conclusion

Preparation and grafting of cultured skin substitutes is a complex process, with high requirements for stringency to be successful consistently. However, the high stringency and special considerations for their routine use result from anatomic and physiologic deficiencies of the cultured grafts. Therefore, it may be expected that the stringency of preparative and grafting procedures for skin substitutes will decrease as graft composition becomes more homologous with native skin. Accomplishment of this goal will best serve all of the biomedical applications of cultured skin substitutes.

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