SERUM-FREE CULTURE OF HUMAN EPIDERMAL KERATINOCYTES AND APPLICATIONS TO SKIN SUBSTITUTES

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ABSTRACT

Cultured skin substiutes offer advantages, including reduced donor sites, fewer surgeries, earlier completion of wound closure, and superior functional and cosmetic results compared to conventional meshed split-thickness skin grafting. Human epidermal keratinocytes (HK) grow rapidly (1 population doubling/day) at clonal cell density (25 cells /cm^2) in biochemically defined culture medium consisting of basal medium MCDB 153 supplemented with EGF (5 ng/mL), insulin (5 mcg/mL), and hydrocortisone(0.5mcg/mL). Primary cultures, or stock cultures, are grown in serum-free medium that also contains 0.5% bovine pituitary extract. After primary culture, HK can be stored indefinitely in liquid nitrogen, or subcultured repeatedly. HK in serum-free medium are combined with a dermal substitute made of collagen and glycosaminoglycan (GAG) that is freeze-dried. Pore size and thickness of the dermal substitute are regulated by temperture of freezing of the liquid collagen-GAG co-precipiate, and by concentration of starting materials in the co-precipitate. A planar surface is added to the porous dermal substitute to restrict cultured HK to the outside surface. The dermal substitute can be stored dry, rehydrated, and populated with cultured human fibroblasts (HK). Cultured skin substitutes are similar histologically to natural human skin in cellular organization and total thickness (<1.0mm). Cellular attachments, including hemi-desmosomes, form in vitro between cultured HK and the dermal substitute. HK-HF- collagen-GAG skin substitutes are evaluated in 2X2 cm full-thickness skin wounds on athymic mice for six weeks. Cultured skin substitutes are not exposed to serum after addition of HK to the dermal substitute,and are pre-incubated in the angiogenic factor, basic Fibroblast Growth Factor (bFGF) for 24 hours before surgery. Controls include murine autograft, human xenograft, or no graft. Measurement of wound contraction shows comparable results between wounds treated with cultured skin substitutes (39% original ginal) size) or with human split-thickness skin (50% original size). Staining of healed wounds for HLA-ABC antigens, demonstrates similar rates of graft acceptance for cultured skin substitutes (7/10) and human splitthickness skin (5/6). Cultured skin substitutes are applied to wounds in a single procedure

in analogy to split-thickness skin graft. Clinical applications for cultured skin substitutes include treatment of burns, plastic and reconstructive surgery, and dermatology.

INTRODUCTION

Uninjured skin serves many physiologic functions including, but not limited to: protection from infectious agents and solar radiation, regulation of moisture flux from the body, regulation of body temperture, and perception of the environment. The physiologic functions of skin are performed by a variety of cells in differentiated epidermis (keratinocytes, melanocytes, Langerhans cells), and in the dermis (fibroblasts, vascular endothelium, leukocytes). Skin loss injuries, such as burns, destroy these structures and functions of skin, and can cause mortality or long-term morbidity. Conventional split thickness skin grafting[1] restores the fundamental functions of skin. However, in large injuries, sufficient amounts of donor skin are not available, repeated surgical procedures for harvesting of donor sites are required, and long hospitalization times occur.

To address these limitations of conventional skin grafting, several kinds of skin substitutes have been developed and tested. Epidermal substitutes include cultured human epidermal keratinocytes[2,3] suction blisters placed on allogeneic dermis[4], and thin epidermal grafts placed onto collagen-based dermal substitutes[5]. Dermal substitutes include allogeneic dermis[4,6] collagen-glycosaminoglycan (GAG) sponges without cells[7] or containing cultured fibroblasts[8], and collagen gels containing cultured fibroblasts[9]. This report summarizes in vitro and animal studies of the combination of a collagen-GAG biopolymer sponge that is populated internally with cultured human fibroblasts, and covered externally with cultured human epidermal keratinocytes grown in serum-free and biochemically defined medium.

MATERIALS AND METHODS

Components of skin Substitutes. Cultured skin substitutes were prepared from separate cultures of human keratinocytes (HK), human fibroblasts (HF), acellular collagen-glycosaminoglycan (C-GAG) membranes. C-GAG membranes were made as described previously[10]. The structure of the C-GAG membrane was optimized for pore size to promote ingrowth of fibrovascular tissue from the wound bed, to allow attachment and growth of HF in membrane pores, and for culture of HK on the membrane surface[11]. Culture media used to prepare cultured skin substitutes are summarized in Table I. a) DMEM medium supplemented with 10% FBS, 10 ng/mL EGF, 5 mcg/mL imsulin and 0.5 mcg/mL hydrocortisone; b) serumfree MCDB 153[3] containing 0.5% (v/v) bovine pituitary extract (BPE), with elevated concentrations of selected amino acids,[12] and c) Biochemically defined MCDB 153 in which the EGF is replaced with 10-100 ng/mL basic Fibroblast Growth Factor (bFGF).

Preparation of skin Substitutes. Sepa-

rate primary cultures of HK and HF cells were established respectively from the epidermis and dermis of a skin biopsy.[13] HK were cultured in serum-free MCDB 153 (Table I, medium "b"), and HF in medium "a" (Table I). Primary cultures were subcultured at an approximate ratio of 1:4 before inoculation onto collagen-GAG substrates (16－18 days after primary isolation). HF were inoculated first onto the porous (internal) side of the collagen-GAG substrate in a dish containing medium "a". After 1-2 days, substrates with HF were washed X 3 with medium "b", and HK were inoculated at a density of 3-5 X 10^5cells /cm^2. HK-HF-collagen-GAG skin substitutes were then incubated for an additional 2-4 days in medium "b". For the final 24 hours before surgical application, the skin substitutes were incubated in medium "c", in which the BPE were replaced with bFGF.

TABLE I Media Compositions.

Medium designation	"a"	"b"	"c"
Basal medium	DMEM	MCDB 153	MCDB 153
Defined Supplements			
bFGF			10-100 ng/mL
EGF	10 ng/mL	10 ng/mL	
insulin	5 mcg/mL	5 mcg/mL	5 mcg/mL
hydrocortisone	0.5 mcg/mL	0.5 mcg/mL	0.5 mcg/mL
Undefined Supplements			
BPE		0.5 % (v/v)	
FBS	10 % (v/v)		

Expermental Treaments. All animal studies were approved by the UCSD Animal Subjects Committee. Cultured skin substitutes (Treatments B, N=10) were compared on athymic mice (Balb/c, nu/nu) to human xenograft (Treatment C, N=16), murine autograft (Treatment D, skin rotated 180°, N=16), or no graft (Treatment A, N=18). Mice were anesthetized with avertin (tri-bromo ethanol in tert-amyl alcohol), and 2 X 2 cm full-thickness skin defects were prepared to a depth leaving the panniculus carnosis intact. Wound perimeters were tatooed at eight points, treatments were administered, 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 Gurad bandages. Data were collected six weeks post surgery.

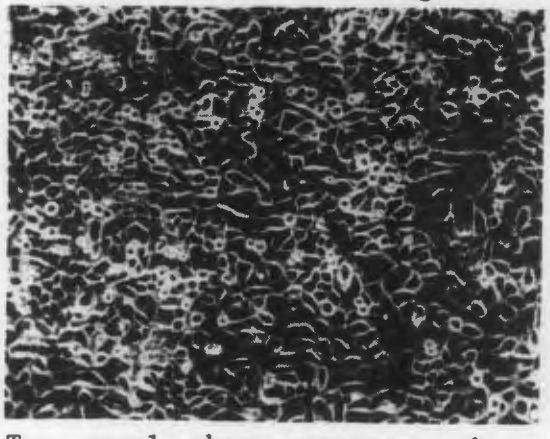
Data Collection and Analysis. Data were collected at six weeks post surgery for analysis of wound contraction and graft acceptance. Wound contraction was determined by rectilinear planimetry of areas bounded by tatoo marks made at the wound margin at the time of surgery. Data for wound contraction are expressed as % Orjginal Wound Size (mean ± SEM)[14]. Pairwise comparison of experimental groups was performed using Student's t Test, and statistical significance was assumed at the 95% confidence level ($p<0.05$). Graft acceptance was determined by positive immuno-fluorescence staining of healed epidermis with fluorescein labeled monoclonal antibodies against a common hapten of the HLA-ABC histocompatibility antigens (Accurate Chemical and Scientific Corp.; San Diego, CA)[15]. Tissue samples for immuno-histochemical staining were prepared from cryostat sections of unfixed, fresh-frozen excised skin from wound

sites. Data for graft acceptance are expressed as % HLA-ABC positive wounds.

RESULTS

Serum-free culture of human epidermal keratinocytes. Cellular morphology and overall efficiency of growth of HK cultured in serum-free medium are shown in Figure 1. Typical epithelial morphology (polygonal cells) and high mitotic rate (sperical refractile cells) are evident (top, Figure 1). Exponential expansion

Figure 1.

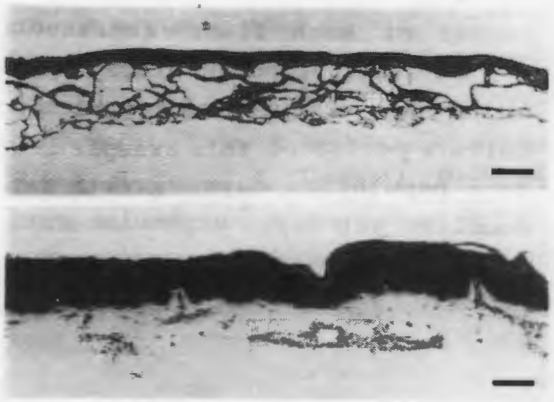


Top panel, phase contrast photomicrograph of human epidermal keratinocytes cultured in serum-free medium MCDB 153 supplemented with 10 ng/mL EGF, 5 mcg/mL insulin, 0.5 mcg/mL hydrocortisone, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, and 0.5% (v/v) bovine pituitary extract. Polygonal morphology of epithelial cells is expressed, and many mitotic cells (spherical, refractile cells) demonstrating the high viabilityof the culture. Scale bar = 0.1 mm. Bottom panel, 75 cm^2 tissue culture flasks covered with sheets of cultured HK cells. Dark spot in the center of each flask represents a 4 mm diameter punch biopsy from which each culture was initidated. Culture period of this example is 18 is 1 population days, growth rate doubling per day, expansion ratio

of area covered by HK cultured in serum-free medium is shown in the bottom panel of Figure 1 where 75 cm^2 flasks are covered with sheets of cultured HK after 18 days of incubation. These cultures were started from 4 mm diameter biopsies represented by the dark spot in the center of each flask. The net expansion in this example is 1:600.

Histology of composite skin substitute and of natural human skin. Representative histology of composite skin substitute composed of C-GAG substrate combined with HK(top panel) is compared to natural human skin (bottom panel) in Figure 2. Total thickness (<1 mm) and histologic organization of the skin substitute is very similar to natural skin with the epithelial component restricted to the external surface. The reticulated surfaces of the C-GAG substrate appear in transverse

Figure 2.

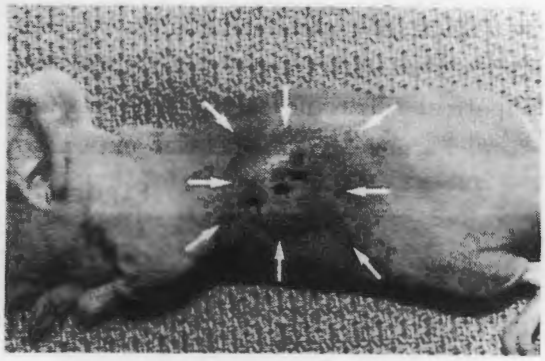


Histological comparison of cultured cell-biopolymer skin substitute and human split-thickness skin. Top panel, composite skin substitute consisting of collagen-glycosaminoglycan dermal substitute covered on its surface with cultured human-epidermal keratinocytes. The skin substitute is approximately the same thickness as split-thickness skin (<1mm), and is compartmentalized with cultured epithelium restricted to the epidermal surface. Scale bar = 0.1 mm.

section as a darkly stained network between open pores occurring infrequently in the substrate. HF (not shown)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 six weeks post surgery. Wounds treated with natural skin (Treatments C and D, not shown) closed most rapidly, by subjective evaluation, with complete and stable closure occurring by two weeks post surgery. Although not quantitated, wounds treated with skin substitutes (Treatment B) appeared to require 3-4 weeks to close completely develop fully keratinized and desquamating epidesmal surfaces. Figure 3 shows a wound six weeks post treatment with an HK-HF-collaen-GAG skin substitute.

Figure 3.



Athymic mouse grafted with HK-HF-collagen-GAG skin substitute. Wound margins (white arrows) identify healed area. Pigmented spots developed (black arrows) from human melanocytes contained in the cultured graft.

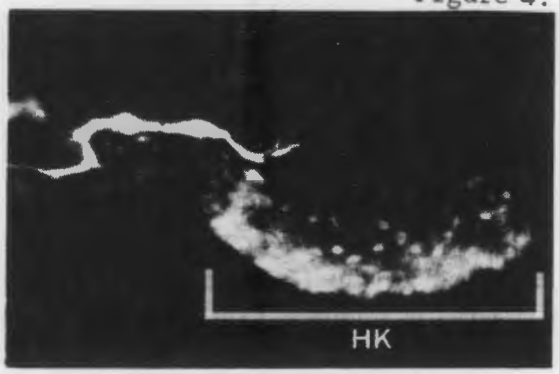
controls (not shown) received no graft, and wounds closed by pronounced contraction and epidermal migration from the wound perimeter to produce typical concave wound margins. Histological examination of wounds closed after treatment with no graft shows a normal epidermal tissue attached to a thin layer of connective tissue over the original woundbed, the panniculus carnosis. Skin substitutes made from C-GAG, cultured human cell and growth factors result in closure of wounds with a more 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. Application of natural human skin results in complete and rapid wound closure. Murine autograft also results in rapid and complete wound closure. Wounds closed with autograft show histologic organization comparable to native murine skin, and no hyperpigmentation.

Wound contraction at six weeks post surgery. Quantitative data collected from rectilinear planimetery of healed wounds is summarized in Table II. Wounds that received no graft (Treatment A) contracted most, to 19.3±1.2% of original size. HK-HF-collagen-GAG skin substitutes in which the HK were not exposed to FBS (Treatment B) showed contraction to 38.5±2.6% original wound size. This value was not different statistically ($p<0.20$) from natural human skin (Treatment C), at 51.7±5.6% of the original size. As expected, murine autograft (Treatment D), contrasted least of any wound treatment, to 66.4 ± 4.2% original size at six weeks post surgery.

Acceptance of grafts containing human cells. Positive and specific staining with fluorescein-labeled monoclonal antibodies 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 keratinocytes. Specific staining for HLA-ABC of epidermis on closed wounds was scored as graft acceptance. Natural human skin stains specifically and uniformly for HLA-ABC. Similarly, acceptance of cultured epithelium produces specific staining (Figure 4) that allows identification of the wound margin where murine and human epidermis meet. Results of scoring of HLA-ABC staining of the experimental treatment are presented in Table II. HK-HF-collagen-GAG skin substitutes in which HK not been exposed to FBS, and that were pre-incubated for 24 hours in biochemically defined medium containing 100 ng/mL bFGF were accepted in 70% of the grafted animals.

Figure 4.



Immunofluorescence micrograph of healed skin stained with fluorescein-labeled monoclonal antibodies against human HLA-ABC antigens. Nucleated keratinocytes of cultured human epidermis (right, "HK") are stained positively for HLA-ABC, and nucleated cells of mouse epidermis (left) are negative.

TABLE II:
Wound Contraction and Graft Acceptance Deta.

TREATMENT	N	% ORIGINAL WOUND SIZE SEM	% HLA-ABC POSITIVE
A) NO GRAFT	18	19.28 ± 1.21	N.D.
B) C-GAG,HF,HK,bFGF	10	38.51 ± 2.64	70 (7/10)
C) XENOGRAFT	16	51.79 ± 5.83	83 (5/6)
D) AUTOGRAFT	16	66.40 ± 4.29	N.D.

DISCUSSION

Cultured skin substitutes offer improvements over conventional skin grafting of reduced donor sites, fewer surgical operations to complete grafting, shorter hospitalization time, and superior functional and cosmetic results. However, to accomplish these objectives cultured skin substitutes must meet the requirements of any temporary skin substitute in control of fluid loss, infection, and scarring, in addition to availability and cost effectiveness.[16] Realization of the advantages of cultured skin substitutes requires accurate preparation in the laboratory and specialized care in the clinic.

Cell distribution and cell viability in cultured skin are highly important to recovery of skin function. Redevelopment of skin tissue occurs primarily from repopulation of a wound with dermal and epidermal skin cells that retain sufficient viability to continue to replicate, and to reform extracellular matrix. For epidermis, this consists predominantly of keratinocytes (95%), melanocytes (3%), Langerhans cells (1%), and other cell types (1%), The grafts described in this report contain predominantly epidermal keratinocytes, although methods for serum-free culture of human epidermal melanocytes[17] are currently being applied to add pigment cells to these grafts. The principal growth supplements in serum-free medium for HK are epidermal growth factor (EGF), and bovine pituitary extract(BPE), Flow cytometry has been used to demonstrate that 10-20% of HK in this medium continue to cycle for at least two weeks after inoculation onto collagen-GAG substrates.[18] Langerhans cells have been shown to be depleted from HK cultures. But because Langerhans cells are bone marrow derived, they may be expected to repopulate epithelial tissue regenerated from cultured skin. In dermis, fibroblasts constitute one major somatic cell type, but reestablishment of micro-vascular supply through growth of endothelium is critical to graft acceptance and persistance. Endothelial cells have not yet been reported to be added to any skin substitute. Therefore, vascular ingrowth must occur from the wound bed. Basic FGF is a powerful angiogenic compound, and also has been shown to replace the mitogenic stimuli of EGF and BPE for cultured keratinocytes[19] and fibroblasts.[20] Therefore, pre-incubation of cultured skin grafts in bFGF for one day befor application maintains viability of keratinocytes and fibroblasts in vitro and also stimulates microvascular ingrowth in vivo from the woundbed to promote graft accepsance.

Current structural and functional deficiencies of all cultured skin substitutes include lack of epidermal adnexi (hair, oil and sweat glands), abnormal pigmentation, and unknown degrees of innervation (touch and temperature sensation) Conventional split- thickness skin grafts, by definition, contain no epidermal adnexi, but do contain melanocytes that populate the dermal-epidermal junction. Proper pigmentation of cultured skin substitutes is important not only for cosmetic appearance, but also for protection from solar radiation. Long-term

studies of occurrance of carcinoma or melanoma in skin regenerated from cultured cells are not yet available. Innervation of skin generated from cultured cells also remains to be studied, but will affect the sensory capacity of the healed wounds. Sensation in skin healed with cultured cells may influence whether reinjury of the grafted area occurs, or is detected.

Autologous grafts of cultured skin cells have been shown to be successful clinically[21], but they require about three weeks to prepare. Cultured allografts by comparison could be made available very soon after injury and allow further reduction of hospitalization. Although allografts of cultured keratinocytes have been shown to reepithelialize partial thickness wounds[22], they have not been shown to persist indefinitely. The rejection of cultured epidermal allografts does not always occur by active destruction of the allogeneic graft, but rather by slow replacement of the graft by host tissue with little or no inflammation[23]. It is likely, therefore, that cultured skin substitutes containing allogenenic cells may serve as temporary biologic skin substitutes until sufficient amounts of autologous grafts can be prepared.

Applications for cultured skin substitutes include skin grafting for burns, plastic surgery, and dermatology, and also as alternatives to animals in safety testing of consumer products and environmental hazards. Principles for preparation of skin substitutes are derived from many academic disciplines, such as cell biology, polymer chemistry, physiology, immunolngy, surgery and dermatology. These principles extend to preparation of other tissue and organ substitutes such as blood vessels, blood, bone, cartilage, endocrine glands and solid organs. Successful development and testing of these tissue and organ substitutes requires comprehensive and interdisciplinary studies. Successful implementation of skin and other tissue substitutes can contribute to new standards for public health and welfare in the future.

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