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Biologic attachment, growth, and differentiation of cultured human epidermal keratinocytes on a graftable collagen and chondroitin-6-sulfate substrate

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Repair of full-thickness burns requires replacement of both the dermal and the epidermal components of the skin. Use of tissue culture methods allows very large expansions of surface area to be covered by cultured normal human epidermal keratinocytes (HK). Porous and resorbable materials, such as collagen and chondroitin-6-sulfate membranes, may be expected to adhere to wounds and promote fibrovascular ingrowth better than grafts of cultured epidermal keratinocytes alone. This article demonstrates the in vitro formation of biologic attachments between HK and a collagen and chondroitin-6-sulfate dermal skin replacement. Dermal membranes are prepared as generic acellular sheets and stored in the dry state for extended periods. Subconfluent HK cultures in logarithmic phase growth can attach quickly to dermal membranes in vitro, form a confluent epithelial sheet on the surface of each membrane, and exhibit mitotic cells for at least 1 week in vitro. Transmission electron microscopy demonstrates the formation of hemidesmosomes, extracellular matrix, and banded collagen at the interface of the epidermal cells and the dermal membrane. By comparison, HK cultures as confluent sheets released enzymatically with Dispase do not attach to the dermal membranes in vitro, under the conditions tested, although complete coverage of the membrane by the cell sheets is obtained. Growth assays show that subconfluent HK cells retain sufficient growth potential to maintain logarithmic phase growth, but that HK cells disaggregated from confluent sheets become growth arrested in comparison. The composite material has discrete dermal and epidermal compartments, has total thickness comparable to split-thickness skin graft, and can be applied to full-thickness skin defects in a single procedure.

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PERMANENT REPAIR OF full-thickness burns ultimately requires restoration of stable tissue as a protective covering on the exterior of the body. Full-thickness burns, by definition, destroy all epidermal and dermal tissue of the skin. Ideally, the repaired tissue would duplicate the structure and function of undamaged skin, including compartmentalization into normally organized epidermal and dermal components. Meshed split-thickness autograft[1] meets these criteria but is often not available in large body surface area (BSA) burns. Contemporary procedures for surgical application of meshed autografts also inflict further injuries to patients and ordinarily accomplish ratios of tissue expansion that are less than 10 times the size of the donated tissue. These limitations in the use of meshed autograft for treatment of large BSA skin loss injuries result in repeated surgical operations, protracted hospitalization, and undesirable cosmetic results.

In response to these limitations, a variety of materials that are obtained from either in vitro or ex vivo preparations have been proposed and demonstrated to function as full (epidermal and dermal) or partial (epidermal or dermal) permanent substitutes

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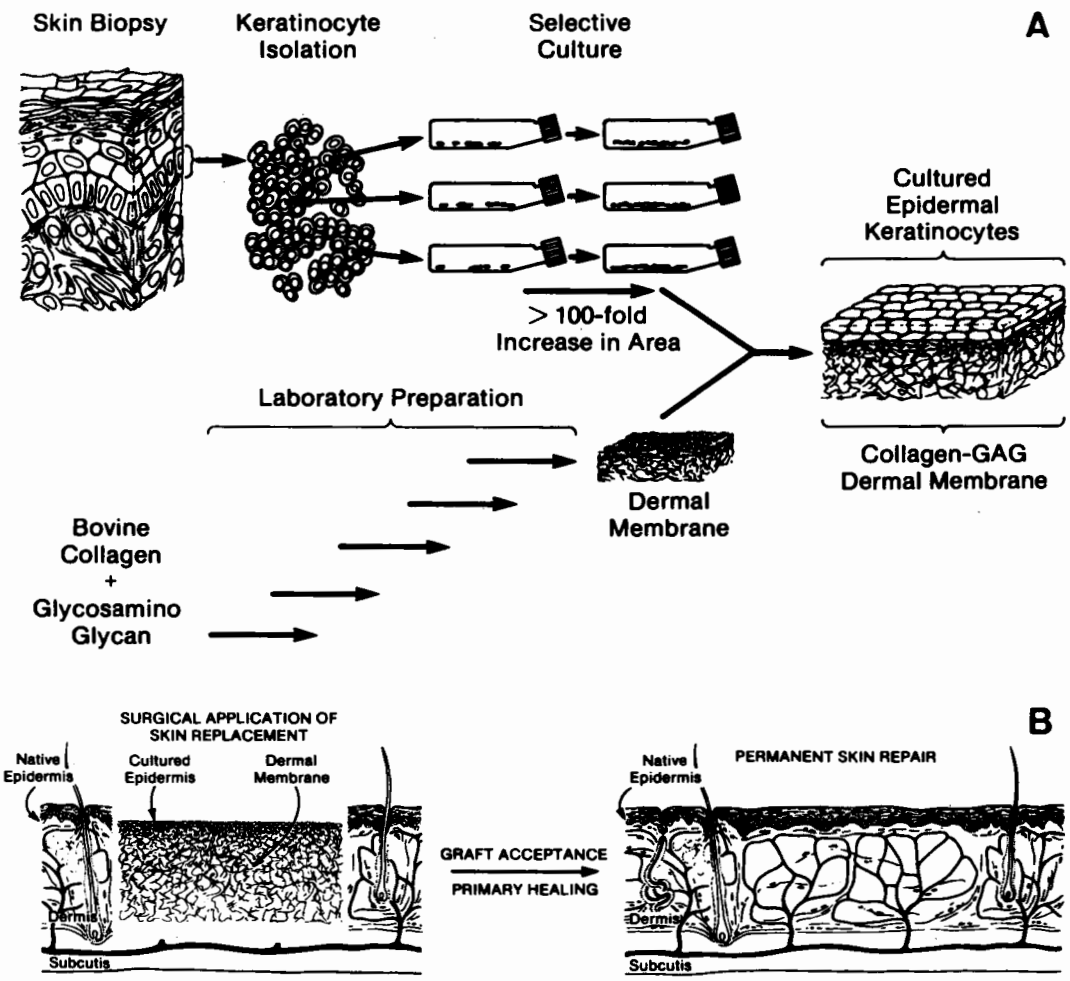

Fig. 1. Diagram of experimental hypothesis. **A,** Composite skin grafts are prepared in vitro from normal HK and collagen-GAG membranes. Dermal membranes are prepared as acellular sheets and stored dry until needed. HK are isolated and placed into selective culture, grown rapidly, inoculated onto rehydrated dermal membranes, and stimulated to stratify. **B,** Composite grafts may be applied surgically onto full-thickness skin defects and promote the restoration of stable skin tissue, including a fully stratified and desquamating epidermis without adnexal structures and a dermal connective tissue that resembles undamaged dermis.

for meshed split-thickness autograft. The use of tissue culture techniques for normal human epidermal keratinocytes (HK)[2-5] can accomplish expansion ratios of area coverage that can exceed 1000-fold in 3 to 4 weeks. HK cultures of these kinds will form multilayered sheets and have been shown to provide wound closure after application to excised full-thickness burns.[6-8] Dermal skin replacement has been demonstrated by application of collagen and chondroitin-6-sulfate (GAG) dermal membranes,[9-11] by allogeneic dermis,[6,12] and by fibroblast-collagen-gel mixtures.[13]

Among the necessary properties of any skin substitutes, including permanent skin substitutes, are rapid and sustained adherence to the wound surface, tissue compatibility, and an inner surface structure that permits ingrowth of fibrovascular tissue.[14] Each of these properties contributes to both the predictability and the rate of graft acceptance onto wounds. Some full (epidermal and dermal) permanent skin substitutes are applied in two stages with de-epidermized allograft applied first to the wound bed followed either by autologous epidermal suction blisters[12] or by autologous HK cultures.[6] Although such approaches may offer distinct advantages over meshed split-thickness autografting, they are subject to availability, variability, and possible biologic contamination of human

Table I. Effects of HK confluency and composite graft submersion on membrane coverage, cell division, and stratification

Inoculum density	*Location in medium*	*N*	*Cellular attachment*	*Mitotic cells*	*Membrane coverage*	*Relative (1+ to 4+) stratification*
Postconfluent	Submerged	3	−	−	Total	++
Postconfluent	Air-liquid	5	−	−	Total	++
Subconfluent	Air-liquid	4	+	−	Partial	++++
Subconfluent	Submerged	35	+	+	Total	+++

allograft. Furthermore, these approaches require two events of graft acceptance to complete wound closure, whereas split-thickness autograft requires only one.

The present study demonstrates the biologic attachment, growth, and differentiation in vitro of HK cultures on collagen-GAG dermal membranes. The keratinocytes grow and stratify to form a continuous epithelial layer over the surface of the dermal membranes, which serve both as a mechanical support for the cell sheets and as a biodegradable connective tissue matrix into which vascular supply and noninflammatory connective tissue can develop from the wound bed. These composite grafts are compartmentalized histologically like skin with the keratinocytes restricted to the external surface of the porous dermal membrane, and they may be prepared with autologous or allogeneic epidermal cells. The formation of biologic attachments in vitro between epidermal cells and the collagen-GAG substrate allows the composite graft to be applied to the wound bed in a single procedure in analogy to split-thickness skin.

MATERIAL AND METHODS

Experimental hypothesis. Composite grafts may be made from separate preparations of HK cultures and acellular collagen-GAG membranes[15] as is summarized diagrammatically in Fig. 1, *A*. Collagen-GAG dermal membranes are prepared as "generic" acellular sheets and may be stored in the dry state for extended periods (at least several months). Shortly before use, membranes are rehydrated and either stored in 70% isopropanol for up to 1 month or placed immediately into tissue culture. Proliferative basal HKs are isolated from tissue, expanded exponentially in number in standard polystyrene tissue culture flasks, and allowed to cover areas that can exceed 1000 times the area of the tissue from which the cells were isolated. Mitotic HK cells attach rapidly to the dermal membranes and become confluent to form a continuous sheet of epithelium. The HK sheet is promoted to stratify and differentiate to a moderate degree, and then it can be applied as a graft with epidermal and dermal components (Fig. 1, *B*). The dermal membrane provides superior graft adherence compared with HK cultures alone and promotes the ingrowth of vasculature and noninflammatory tissue from the wound bed. The cultured epidermis fuses with undamaged epidermis from the wound margin to reestablish a continuous epidermal cover that can provide permanent wound closure. The resulting restoration of connective tissue and epidermis can hypothetically fulfill the need for stable tissue to repair the wound. This report contains evidence to support the first half of this hypothesis.

Formation of dermal membranes. Dermal membranes were made according to the methods of Yannas et al.[16] with modifications. To summarize, comminuted bovine collagen was partially solubilized in 0.05 mol/L acetic acid and co-precipitated with chondroitin-6-sulfate in a refrigerated homogenizer. The co-precipitate was cast into sheets and frozen on the refrigerated shelves of a Virtis Unitop 800 lyophilizer manifold (The Virtis Company, Inc., Gardiner, N.Y.). Shelf temperature was regulated within ±1° C by a digital temperature controller and monitored with a metallic temperature probe frozen to each shelf. Temperature probes were calibrated to 0° C with water that contained melting ice. The frozen co-precipitate was lyophilized overnight and subjected to vacuum dehydration at 105°C and 1×10^{-4} torr for 24 hours. Dry membranes were rehydrated in 0.05 mol/L acetic acid for 24 hours, cross-linked in 0.25% glutaraldehyde for 24 hours, washed exhaustively in purified water (Millipore Milli-Q, 18 megohm-cm resistivity, Millipore Corp., Bedford, Mass.), and stored in 70% isopropranol. Membrane structure was regulated as reported elsewhere.[17]

Tissue culture conditions. HK cultures were initiated from surgical discard specimens and cultured in nutrient medium MCDB 153 containing 0.3 mmol/L calcium,[2-4] increased amounts of selected amino acids,[7] 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.1 mmol/L ethanolamine, 0.1 mmol/L phosphoethanolamine, 0.5% vol/vol

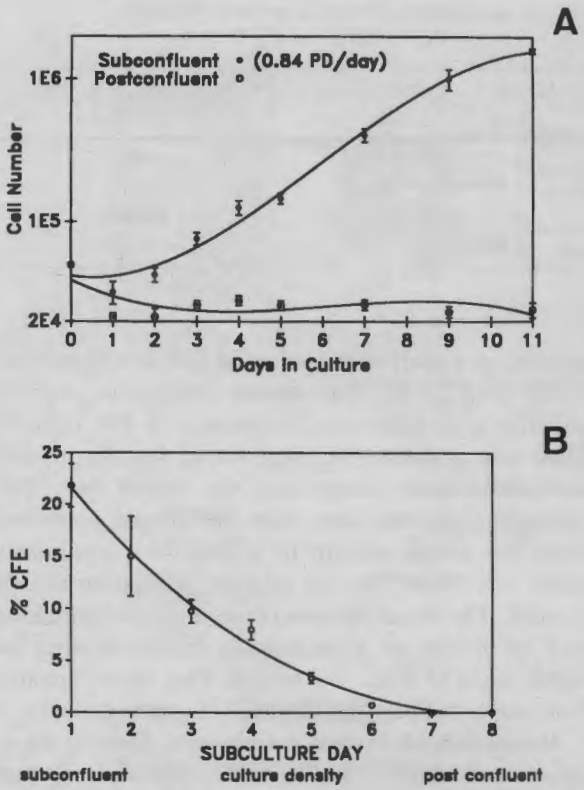

Fig. 2. Effects of confluency on HK growth rate and colony-forming efficiency. Assays performed in biochemically defined culture medium as described in Material and Methods. **A,** Subconfluent HK cells *(solid circles)* proceed into classic sigmoid growth after inoculation, but HK cells from confluent cell sheets *(open circles)* show no increase in cell number after 11 days of incubation. Maximum growth rate for subconfluent HK cells is 0.84 population doublings per day. **B,** Daily inoculation from parallel cultures, beginning with subconfluent HK cells, shows a steady decrease in CFE from 21.8% for a subconfluent inoculum to 0% for a postconfluent inoculum.

bovine pituitary extract, and penicillin-streptomycin-amphotericin B (Gibco Laboratories, Grand Island, N.Y.) as an antibiotic-antimycotic agent.

Assays of HK growth and colony formation. Growth assays were performed by inoculation of 5×10^4 HK cells from subconfluent (exponential phase) or confluent (stationary phase) HK cultures into 60-mm Petri dishes (Lux #5220, Miles Scientific, Naperville, Ill.). After 1, 2, 3, 4, 5, 7, 9, and 11 days of incubation, the cells were harvested, counted, and cell numbers were plotted versus time in days. Colony-forming efficiency (CFE) was determined as previously described[4] with a colony defined as eight or more contiguous cells after an incubation period of 12 days.

Fig. 3. HK and collagen-GAG composite graft. Grafts can be readily handled, applied, and relocated without enzymatic treatment or damage to the epidermal surface.

HK cells (500 cells/60 mm dish; 25 cells/cm^2) from parallel stock cultures were inoculated daily, beginning before confluency and ending after confluency. Data points for all assays are expressed as mean plus or minus standard error of the mean of triplicate dishes from duplicate assays.

Inoculation of dermal membranes with HK. Dermal membranes, 7 cm in diameter, were transferred sterilely from 70% isopropanol to Petri dishes that contained Hepes-buffered saline solution (three changes) followed by tissue culture medium (two changes, minimum of 1 hour each) as described above or, as above, plus 20% vol/vol fetal bovine serum (FBS). Membranes were submerged in the media or elevated to the air-liquid interface[18] on stainless steel supports (4 mm high × 82 mm diameter) in 100 mm glass Petri dishes. HK in logarithmic phase growth were removed from flasks by trypsinization and inoculated onto the membranes at a density of 0.5 to 1×10^6/cm^2. After 5 to 11 days incubation, the HK-dermal membrane composites were fixed in buffered glutaraldehyde and prepared for microscopic examination.

Histology and electron microscopy. Dermal membrane–HK composites were embedded in glycol methacrylate (JB-4; Polysciences, Inc., Warrington, Pa.), sectioned at 5 μm thickness, and stained with 0.1% toluidine blue for light microscope histologic examination. Identical specimens were embedded in epoxy resin, which was followed by preparation of ultrathin sections, staining with uranyl acetate and lead citrate, and examination by transmission electron microscopy (TEM).[19]

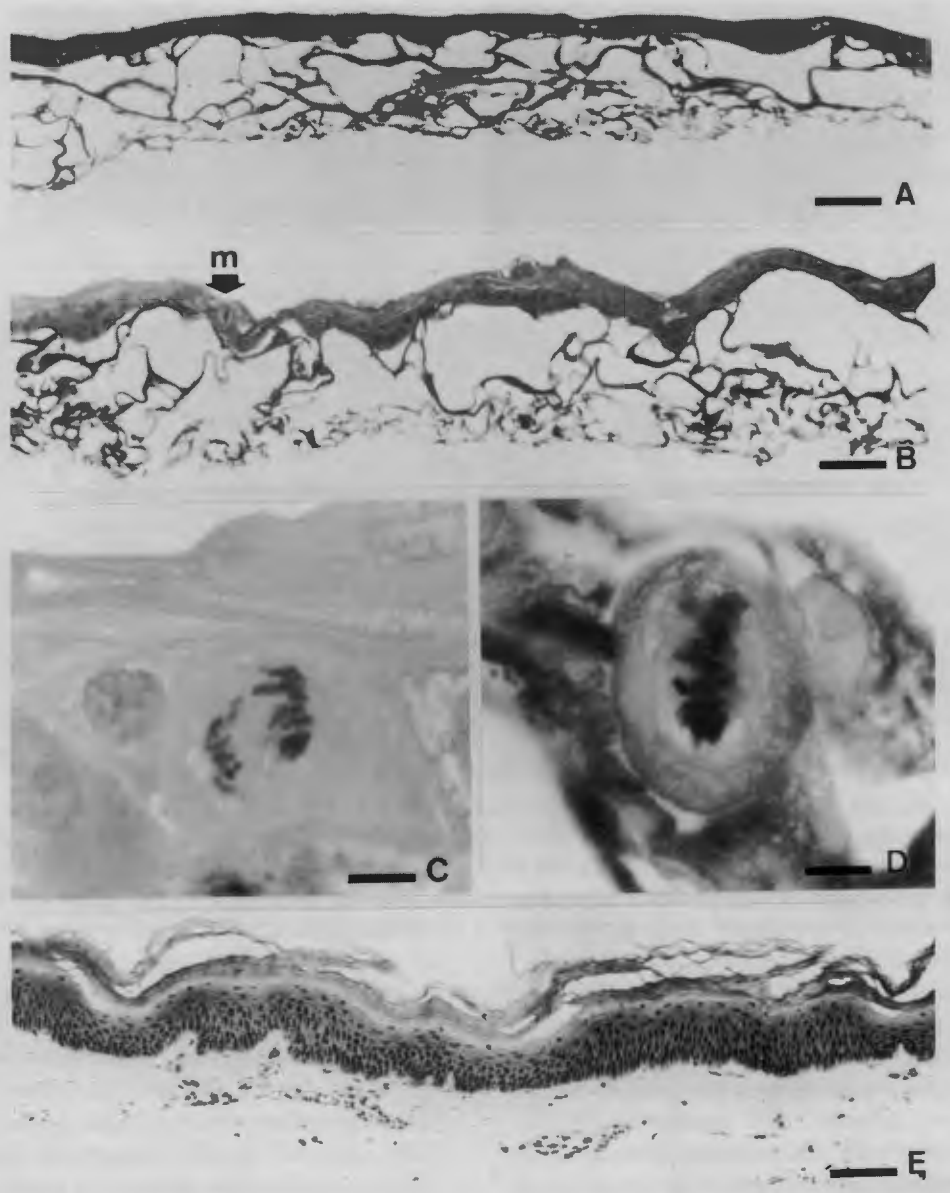

Fig. 4. Photomicrographs of composite grafts in vitro and of split-thickness skin. **A,** Composite graft after 5 days' incubation in culture medium containing 20% vol/vol FBS. HK form a partially stratified epithelial layer with uniformly flattened cells on the external surface. HK are located only on the surface of the dermal membrane. **B,** Composite graft after 5 days' incubation in serum-free culture medium shows a mitotic cell (*m*, enlarged in **D**). Similar histologically to **A,** but superficial cell layers are less well organized into epidermal strata. **C,** and **D,** HK growth, in vitro, on the collagen-GAG membrane continues after 5 days as confirmed by the presence of mitotic cells in growth medium containing FBS (**C,** and **A**) and without FBS (**D,** from **B**). **E,** Split-thickness human skin. (**A, B, E,** scale bars = 100 μm; **C, D,** scale bars = 10 μm.)

RESULTS

HK attachment, mitosis, and differentiation on collagen-GAG membranes. Table I summarizes the attachment, mitosis, and stratification of subconfluent and confluent HK-culture cells on dermal membranes either submerged in culture medium or elevated to the air-liquid interface[18] for 11 to 14 days. All results were reproducible for the number of samples *(N)* shown.

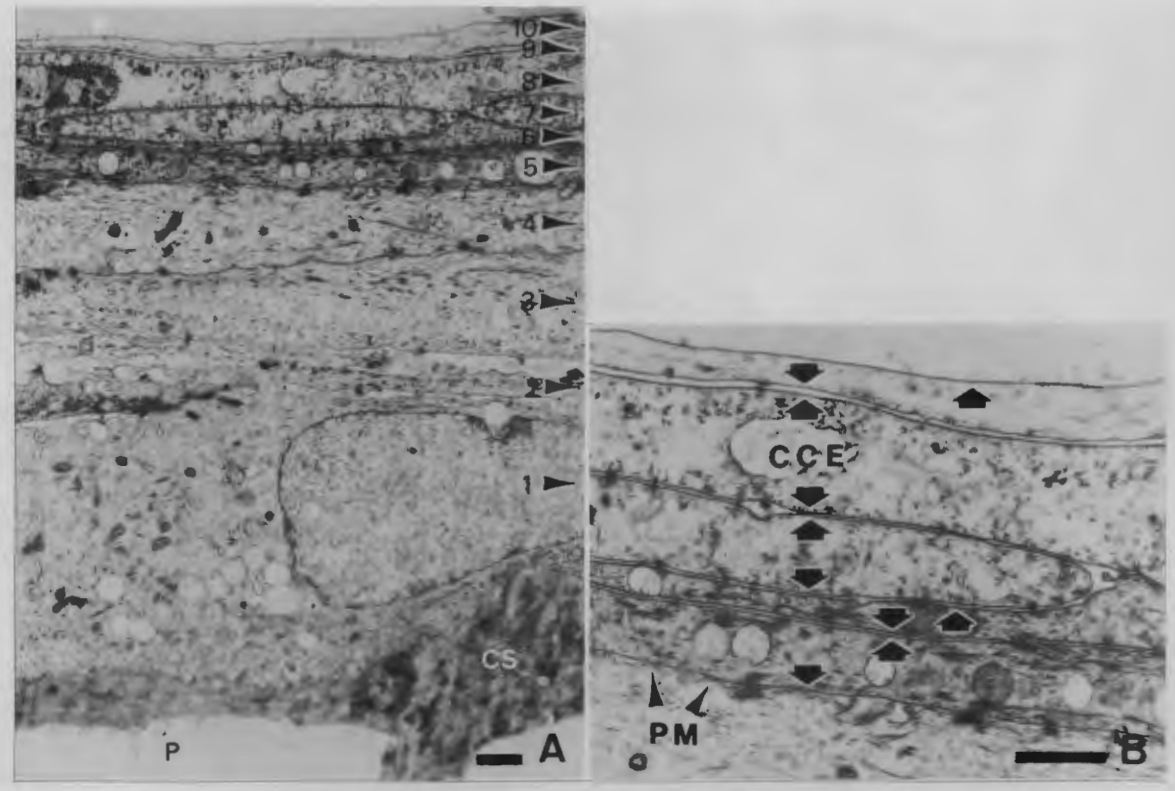

Fig. 5. Transmission electron micrographs of composite graft after 11 days in culture. **A,** *Bottom to top,* pores *(P)* in the collagen substrate *(CS)* occur very close to the substrate surface. HK cells attached to the substrate are less flattened than HK strata not directly attached to the substrate. Numbers on the right edge of the micrograph identify 10 HK strata. **B,** Uppermost HK strata develop cornified cell envelopes *(CCE)* that appear as thickened cell peripheries, compared with the uncornified plasma membranes *(PM)* seen in the intermediate and lower HK strata. (Scale bars = 1 μm.)

Subconfluent cultures attach to the membranes within 24 hours, whether submerged or elevated. However, only submerged cell-membrane composites became completely covered with HK cells that were partially stratified, whereas elevated composites grown in parallel resulted in partial coverage of the membrane surface but were more completely stratified. Confluent HK sheets released with the neutral protease, Dispase,[5] failed to attach to dermal membranes whether submerged or elevated, showed no mitotic cells, and showed no change in the degree of epidermal stratification during culture periods up to several weeks after the time of enzymatic release. To identify the basis for differences of attachment to dermal membranes of actively growing HK and of HK cell sheets, assays of HK cell growth and CFE were performed with single cell suspensions from subconfluent HK cultures and from confluent HK cell sheets.

HK growth and colony-forming efficiency. Growth of subconfluent and confluent HK cells is shown in Fig. 2, *A*. After inoculation into defined culture medium, subconfluent (exponential phase) HKs proceed into the exponential phase of growth (0.84 population doublings per day), but confluent (stationary phase) HKs are growth arrested and show no significant increase in cell number. CFE of HK cultures as they become confluent is shown in Fig. 2, *B*. CFE decreases from 21.8% in subconfluent cultures to 0% in postconfluent cultures as have been used for grafting.[5-7]

Physical qualities of grafts. Composite grafts of cultured HK and collagen-GAG membranes can be handled without secondary supports or transportation vehicles (Fig. 3). Grafts may be draped into place, lifted, and relocated without damage to the cultured epidermal surface, and they are less fragile than cultured epidermal sheets alone.

The dermal membrane alone has limited elasticity

and tensile strength by subjective evaluation. After the formation of a partially stratified epidermal layer on the membrane surface, the physical strength of the composite graft increases in correspondence to the degree of stratification of the epidermal layer. Subsequent to the formation of an epidermal layer in vitro, the composite graft has greater physical strength than do cultured epidermal sheets alone or dermal membranes alone, but less than split-thickness skin. Although the strength of the composite graft exceeds that of cultured-cell sheets alone, securing of the composite graft is enhanced by suturing of both it and the overlying dressing to the wound margin. Placement of suture through undamaged skin at the wound margin, into underlying tissue, and out through the cultured graft and overlying dressing is effective in the immobilization of the graft to the wound bed.

Histology. The histologic appearance of composite grafts is compared with that of split-thickness skin in Fig. 4. Five days after inoculation with cultured HK, a continuous and partially stratified epithelial layer has formed. The addition of 20% fetal bovine serum (FBS) for at least 48 hours promotes the formation of a uniform HK layer, which contains cuboidal cells attached to the collagen-GAG membrane and flattened cells on the external surface (Fig 4, *A*). An identical sample without FBS (Fig. 4, *B*) also exhibits cuboidal cells attached to the dermal membrane but somewhat less well-organized "suprabasal" HK layers. Histologic examination of both conditions showed discrete separation into epidermal and dermal compartments and mitotic cells adjacent to the dermal membrane (Fig. 4, *C* and *D*). The discrete dermal and epidermal compartments compare favorably with the histologic organization of split-thickness skin (Fig. 4, *E*).

Thickness and pore size of the dermal component of the graft result from regulation of the preparative procedure by which the dermal membrane is made. In this study these procedures were regulated to generate a graft with a total thickness, including epidermis, of less than 0.5 mm (0.020 in). Composite grafts in Fig. 4, *A* and *B*, range in thickness from 0.2 to 0.3 mm (0.008 to 0.012 in). The procedure for preparation of the membrane are highly reproducible and can be regulated deliberately within narrow limits to generate dermal membranes with predictable characteristics of thickness and pore size. More detailed descriptions of the preparative procedures and quantitative data to support the principles by which the structural composition of the dermal membrane is regulated are presented elsewhere.[17]

Transmission electron microscopy. Descriptions of TEM of composite grafts refer to "dermal membranes" as "collagen substrates" to avoid confusion of "dermal membranes" with "plasma membranes" of HK cells contained in the composite grafts. HK cultures on collagen substrates form stratified, partially cornified epidermal layers after 11 days in culture (Fig. 5). At low magnification, 10 cell layers are seen (Fig. 5, *A*), and cells attached to the collagen substrate are much less flattened than are stratified cells not attached directly to the substrate. Only the uppermost strata exhibit cornified cell envelopes (CCE, Fig. 5, *B*). Desmosomes occur frequently (Fig. 6) to connect the cell layers and confirm the epithelial nature of the cultured cells. At the interface of the cultured HKs and the dermal membrane, cellular attachments to the membrane are observed. Hemidesmosomes (Fig. 7, *A* and *B*) form between HK cells and the collagen substrate to provide cellular adhesions to the substrate. Extracellular matrix (ECM, Fig. 7, *A* and *B*) is located continuously between HK and the membrane. The composition of this ECM has not been determined in this study, but HK cells grown in these culture conditions have been shown to deposit fibronectin on the substrate to which the cells attach.[20] Hemidesmosomes and ECM are distributed uniformly along the cell-substrate junction both in serum-free samples and in those with 20% FBS. In addition, composite grafts exhibit extracellular fibers in the collagen substrate immediately adjacent to the cell-substrate junction

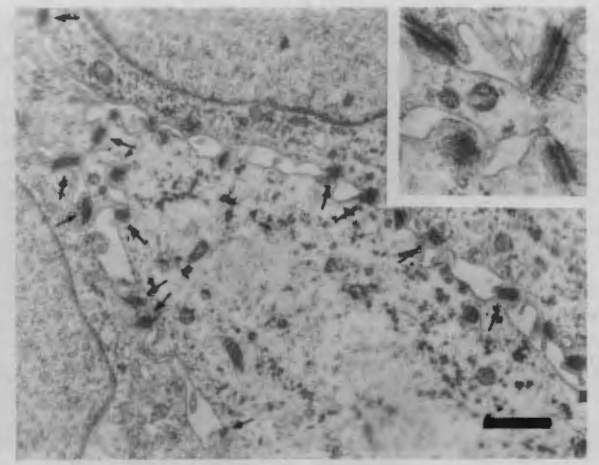

Fig. 6. Transmission electron micrographs of desmosomes that interconnect HK in culture. *Arrows* point to desmosomes that form frequent biologic junctions between HK cells in vitro and confirm the epithelial nature of these cultured cells. **Inset,** Enlargement shows detail of desmosomal junctions between HK cells. (Scale bar = 1 μm.)

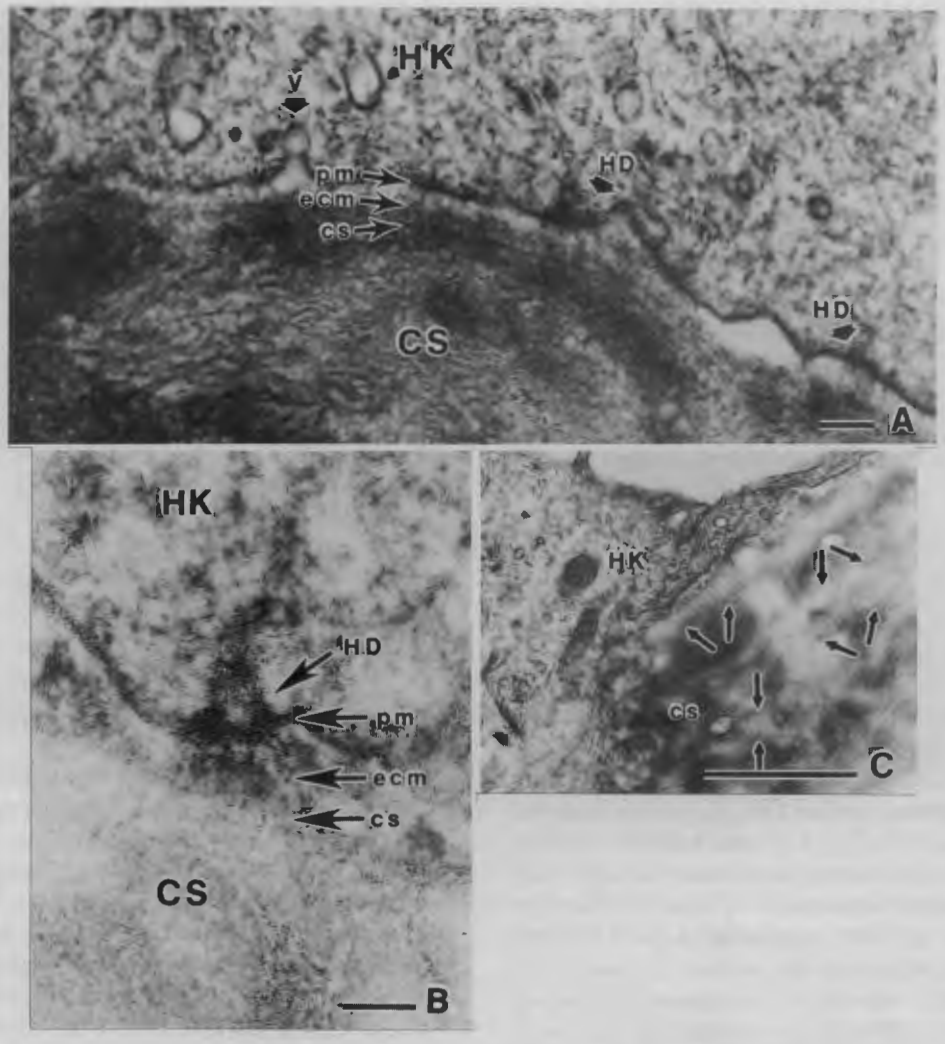

Fig. 7. Transmission electron micrographs of biologic attachments between HK and collagen substrate. **A,** *HK* cell attaches to the collagen substrate *(cs)* by hemidesmosomes *(HD)* and formation of extracellular matrix *(ecm)*. Hemidesmosomes provide local cellular adhesions beginning in the cytoplasm, crossing the plasma membrane *(pm)* and extending through the extracellular matrix to the collagen substrate. Extracellular matrix is found continuously between the HK and the substrate. Vesicles *(V)* in the HK plasma membrane may participate in an exocytotic pathway for deposition of the extracellular matrix. **B,** Hemidesmosome structure that forms biologic attachment between *HK* and collagen substrate *(CS)* involves the *HK* plasma membrane *(pm)* and extracellular matrix *(ecm)*. **C,** *Arrows* point to fibers in the collagen substrate with a repeating pattern that is consistent with the periodic register of collagen. These fibers were found only immediately adjacent to the cell-substrate junction, which implies their deposition by *HK* in culture. (Scale bars = 1 μm.)

(Fig. 7, *C*). The repeating pattern in the fibers is consistent with the periodic register of collagen. These fibers have been found only within short distances of the cell-substrate junction, which implies their deposition by HK in culture. The collagen type of these fibers was not determined in this study.

DISCUSSION

Skin replacement after large BSA burn injuries remains a complex and unsolved problem. Factors requiring consideration for graft success include the characters both of the graft and of the wound bed. Unmeshed split-thickness autograft provides the high-

est probability of acceptance and persistence on the wound and high quality of repair for skin-loss injuries, including excised full-thickness burns. However, even an optimal graft of this kind may be only partially accepted on a suboptimal wound bed. Factors that influence greatly the characters of the wound bed and graft acceptance include, but are not limited to, depth of excision, corresponding type of tissue in the wound bed, and presence of infection.[21,22]

Similarly, differences in graft acceptance may be expected with the applications of grafts of various compositions to an optimal wound bed. In comparison with split-thickness autograft, biologic materials (fresh or frozen allograft dermis, fresh or processed porcine xenograft, collagen-based dermal implants, cultured HK sheets) may be expected to accomplish wound closure, in part, according to their respective capabilities to support fibrovascular ingrowth. Cultured HK-cell sheets, because of the intrinsic lack of vascular supply in epidermis, may be expected to accept onto wounds in correspondence, predominantly, with the degree and quality of vascularized connective tissue in the wound bed. Acceptance of 60% to 80% of HK-cell sheets onto "healthy beds of granulation tissue" after allograft removal has been reported,[8] but the acceptance of cultured HK grafts onto freshly excised full-thickness burns has had little success. Furthermore, although granulation tissue may serve as a satisfactory wound bed for acceptance of cultured HK and other grafts, it is also associated with the formation of scar.[23]

Normal skin is compartmentalized into epidermal and dermal components, and split-thickness skin grafts are usually less than 0.5 mm (0.020 in) in thickness. To approximate the dimensions of split-thickness skin grafts, the thickness of the dermal membrane is deliberately controlled to be less than 0.5 mm. Compartmentalization of the cultured epidermis to the surface of the porous dermal membrane requires a pore size at the surface that is less than the diameter of a cultured cell (15 to 20 μm), because attachment, growth, and differentiation of the cells are enhanced by inoculation of single cell suspensions of confluent keratinocytes. However, ingrowth of connective tissue and vascular supply are enhanced by pore size of at least 80 μm.[14] Therefore the pore size of this dermal membrane is regulated to from 50 to 150 μm to promote tissue ingrowth. Earlier work at this laboratory[15] showed that this range of pore sizes also allowed the invasion of the membrane by cultured keratinocytes inoculated onto its surface. This problem was solved by the addition of a thin, nonporous layer of collagen and chondroitin-6-sulfate to one surface of the dermal membrane. The nonporous layer restricts the cultured cells to the external surface of the porous membrane, which results in the compartmentalization of the cultured epithelium to the membrane surface.

The porous and resorbable dermal membrane described here provides an alternative mechanism for adherence of cultured HK cells to the wound bed—primarily by entrapment of coagulum from the wound and secondarily by ingrowth of vasculature and noninflammatory tissues from the wound bed. Better adherence of and fibrovascular growth into the dermal membrane of this composite graft may be expected to contribute to more rapid wound healing compared with cultured epidermal grafts alone, but confirmation of this postulation requires further study. Formation of biologic attachments between cultured HK and the dermal membrane allows for a single event of adherence of the dermal-epidermal cultured graft to the wound, rather than two separate events of adherence for individual dermal and epidermal components. Cellular attachments also allow the membrane to function as a transportable culture substrate and thereby to eliminate the need for enzymatic release of the cultured epidermal cells from plastic tissue culture substrates before grafting. Determination of the molecular and antigenic compositions of the cellular attachments will be addressed in future studies.

Success of grafts that contain cultured HK also depends on the ability of the cultured cells to continue to proliferate indefinitely in analogy to epidermal stem cells. As shown here, cultured HK sheets[5,7] fail to attach to dermal membranes and have greatly reduced growth potential compared with subconfluent cultures. This finding is consistent with the "density inhibition" of confluent HK cultures as they proceed from exponential growth phase (actively growing, subconfluent) into stationary growth phase (growth arrested, confluent sheets).[24,25] Failure of HK-cell sheets to attach to the membranes is postulated to reflect growth arrest after transition from exponential to stationary phases of cell growth. Conversely, HKs in the exponential phase not only retain growth potential, as demonstrated by growth assays and by the presence of mitotic cells in the composite grafts, but also are capable of producing hemidesmosomes, extracellular matrix, and collagen for attachment to the collagen-GAG membrane. The retention of growth potential by preparation of composite grafts with HK cells in the exponential growth phase may be expected to increase the proportion of cells in the graft that are capable of

long-term proliferation after application to the wound, in analogy to keratinocyte stem cells in undamaged epidermis. These results also predict that application of subconfluent HK cultures may result in more rapid wound closure and in increased rates of graft acceptance than has been demonstrated with applications of confluent HK-cell sheets.

Long-term proliferation is of central importance to the permanence of the cultured graft, but some epidermal stratification is also desirable because it reduces graft fragility and helps protect the proliferative cells from mechanical damage. The addition of FBS to the culture conditions promotes epidermal stratification and differentiation but also allows continued cell division as demonstrated by the presence of mitotic HK within the partially stratified epidermal component of the composite graft. However, FBS has been shown to greatly inhibit the growth of HK in vitro.[25] Reduced growth and increased differentiation in the presence of FBS are comparable to the regulation of HK growth rate and differentiation by calcium ion concentration in serum-free and biochemically defined culture media.[4, 24, 25] Therefore the degrees of continued HK proliferation and HK stratification may be adjusted as functions of the concentrations of FBS and calcium ion in the culture medium. However, the effect of density inhibition of HK proliferation after confluency on the dermal membrane will reduce cellular growth rate independent of the effects of FBS or calcium ion concentrations.

Although cultured autologous epidermal grafts provide a distinct advantage for reduction of tissue donation by the patient to complete wound closure, valuable time is required to grow cultured autografts. This incubation period could be eliminated by the use of cultured allografts, but their potential for rejection remains unresolved. Earlier studies had shown that class II transplantation antigens (HLA-DR) are not expressed by HK after culture for several days,[26] and therefore that cultured epidermal allografts should not be rejected. However, more recent findings have shown that HK cultures will reexpress HLA-DR after exposure to gamma interferon[27-29] or to supernatant culture medium from stimulated peripheral blood mononuclear cells.[27] Therefore the possibility exists that reexpression of HLA-DR on cultured HK after transplantation could occur after a nonspecific activation of the immune system of the recipient and initiate rejection of the allograft. However, graft rejection may also depend on the relative degree of HLA-DR expression by HK compared with its expression by immune system cells, including mononuclear cells and Langerhans cells, from the allogeneic donor. In vitro, maximum HLA-DR expression by HK is less than 20% of the relative expression by stimulated mononuclear cells,[27] and donor immune system cells do not survive in culture. Therefore, the question arises of whether the level of HLA-DR expression by cultured HK, in the absence of donor immune system cells after transplantation, is sufficiently great to initiate graft rejection. If transplanted HK cannot express sufficient levels of HLA-DR to initiate graft rejection under conditions of maximum nonspecific stimulation of the recipient's immune system, then allogeneic transplantation of HK cultures for skin replacement may be safe and permanent.[30] The continuous availability of HK cultures for allografts would eliminate completely the time of incubation required for culture of HK autografts and provide even greater medical advantages in the treatment of full-thickness burns. The dermal-epidermal composite graft described here can be used for further investigations with cultured HK allografts, for clinical applications with cultured HK autografts, and for studies of skin biology and pathology.

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