Cultured Epithelial Autografts: Skin Regeneration and Wound Healing
A Long-Term Biospy Study

Carolyn C. Compton, M.D., Ph.D.
Departments of Pathology, Massachusetts General Hospital
and Shiriners Burns Institute
Boston, MA U.S.A.

Summary

In an 8-year study of 35 patients treated with cultured epithelial autografts (CEA) grafted to full-thickness burn wounds excised to muscle fascia, overall take rates of CEA averaged 55–60%. Biopsies of successfully engrafted CEA were analyzed by light microscopic, immunohistochemical, morphometric, electron microscopic and ultrastructural immunolabelling techniques in order to study skin regeneration and wound healing in these patients. Controls consisted of both normal site- and age-matched skin and healed meshed split-thickness autograft (MSTA) interstices on the same patient biopsied at comparable postgrafting time points.

Key events in the regeneration of skin from CEA can be summarized as follows:

At transplantation, CEA are undifferentiated and lack both granular and cornified cell layers. By 6 days postgrafting, CEA differentiate all normal epidermal strata but lack rete ridges. De novo formation of a confluent basal lamina and mature hemidesmosomes is completed by about 3 weeks. Anchoring fibrils are sparse and morphologically immature compared to normal skin until about 6–12 months postgrafting, but their maturation rate is identical to that of MSTA interstice controls. Hyperproliferation of the newly differentiated epidermis as judged by increased numbers of Ki67-positive (i.e., cycling) cells within the basal layer continues for 4–6 months after grafting. Expression of hyperproliferative keratins (cytokeratins 6/16) continues throughout the first postgrafting year and may be seen up to two years after transplantation. The site-specific phenotype of the donor skin from which the cultures are grown is re-expressed by the CEA shortly after transplantation and is maintained long-term. CEA develop rete ridges and a neodermis with normal stromal and vascular organization at about 6–12 months, whereas MSTA interstice controls do not. At 4–5 years, elastin expression is also observed in the CEA neodermis, completing the dermal regeneration process. Normal epidermal differentiation is maintained long-term, and no epidermal atypia, dysplasia or atrophy is observed. The long-term results indicate that CEA regenerate a stable normal epidermis and are capable of inducing dermal regeneration from immature wound bed connective tissue.

More recent studies on 10 patients in which CEA were transplanted to engrafted, cryopreserved homograft dermis instead of granulation tissue showed increased take rates of CEA (average 85–90%) and acceleration of rete ridge formation and normalization of keratin programs within the differentiated epidermis on histological examination.
Introduction

In the late 1970's, an answer to the problem of limited autograft availability in massive burns was suggested by the development of a new biotechnology that permitted rapid propagation of human keratinocytes in cell culture to form epithelial sheets suitable for grafting\(^1\). Although the idea of using cultured epidermal cells as skin grafts was not new, prior investigations were severely hampered by methodological limitations\(^2-6\). By 1975, however, techniques allowing serial subcultivation of disaggregated human epidermal cells had been developed\(^6\). Successful transplantation of cultured autologous keratinocytes had already been achieved in animal models\(^1,8\), and by 1980, cultured human allografts had been successfully transplanted to immunodeficient mice\(^9\). Further modifications and refinements of culture techniques made it possible to generate enough epidermis to cover the entire human body surface within 3–4 weeks from a small skin biopsy.

Our clinical pilot studies first demonstrated that cultured epithelial autografts (CEA) could be used in the treatment of patients with 3rd degree burn wounds and that permanent wound coverage with CEA was possible\(^10,11\). In 1984, we showed that the use of CEA could be life-saving in pediatric patients with massive burn wounds\(^12\). Thereafter, CEA began to be used for burn wound treatment by other clinical investigators in the United States, Europe, Australia, and Japan\(^13-27\). In 1988, CEA prepared by the method of Rheinwald and Green became commercially available (BioSurface Technology, Inc., Cambridge, MA), vastly expanding the scope of application of this therapy.

The growing importance of CEA in an ever increasing number of clinical settings necessitated a more complete understanding of CEA biology. In order to study the biology of skin regeneration and wound healing following CEA transplantation, we analyzed biopsies from patients treated with CEA for full-thickness burn wounds at various intervals after grafting\(^28\). The aims of this investigation have been twofold: 1) to provide critical, objective evaluation of this important new form of therapy; and 2) to use this unique biological system to study issues of basic scientific interest in the domain of epithelial biology and wound healing.

Materials and Methods

Biopsies from 35 patients (age range: 1 to 59 years) treated with CEA for massive (> 70% body surface area) burn wounds. In most cases, CEA were derived from skin of axillary folds, buttock folds, groin creases, soles of feet or scalp. All CEA were grown by the method of Rheinwald and Green\(^6\) (Fig. 1). CEA were biopsied at varying intervals postgrafting ranging from 5 days to 8 years. Biopsies were derived from areas of successful CEA engraftment ("take") having the best clinical (cosmetic) appearance and were analyzed by histologic, morphometric, immunohistochemical, electron microscopic and ultrastructural immunolocalization techniques. Postgrafting events in epithelial differentiation, epithelial-stromal interactions, dendritic cell repopulation and function, and stromal wound healing were analyzed in comparison with controls of two types: 1) native skin (age-, body site-, and sex-matched normal skin); and 2) epithelial wound healing controls consisting of re-epithelialized interstices of meshed split-thickness autografts (MSTA) from the same patient at comparable postgrafting time points. Both CEA and controls were transplanted to full-thickness wounds surgically excised to muscle fascia immediately following the injury.

More recently, we have studied 10 patients (longest Fig. 1 CEA Production Method
follow-up: 2.5 years) on whom CEA were transplanted to the granulation tissue in full-thickness wounds in some sites and to engrafted, de-epidermized cryopreserved homograft in other sites (all grafted sites being full-thickness wounds). The CEA placed on the two different types of stroma were biopsied at comparable postgrafting time points. Using the CEA transplanted to granulation tissue as the control for comparison, the effect of a mature connective tissue substrate (dermal extracellular matrix) on the CEA could be determined.

Results

Epidermal Differentiation

Grossly, the confluent sheets of cultured keratinocytes that comprise CEA appear translucent and have a mucoid consistency (Fig. 2). They are clipped to a backing of vaselin-impregnated gauze to give them enough structural support to be handled surgically, but they are fragile and must be transferred to the wound bed (after achieving complete hemostasis) with as little manipulation and trauma as possible. Microscopically, they appear as unevenly stratified sheets of keratinocytes (about 3–9 cells layers thick) lacking both granular and cornified cell layers (Fig. 3). Differentiation occurs rapidly after transplantation, and within 5 days, CEA grossly resemble normal epidermis (Fig. 4). Microscopically, a hypertrophic, normally stratified epidermis lacking rete ridges is seen (Fig. 5). With the differentiation of granular and cornified layers within a few days after transplantation, CEA become competent to serve as a barrier to fluid loss and bacterial invasion.

Epidermal maturation can be mapped using antibodies to markers of keratinocyte differentiation including: involucrin\(^{29}\); filaggrin\(^{30}\); CK1/CK10 (the major cytokeratin pair in suprabasal cells of normal epidermis)\(^{12}\); AE1/AE3 (antibodies to keratin subclasses with mutually exclusive expression domains in the normal epidermis)\(^{31,32}\); CK5/CK14 (expression normally limited to basal cells but expressed suprabasally in hyperproliferation states), and CK6/CK16 (not expressed by normal epidermis but produced in hyperproliferation states)\(^{33}\). In vitro, involucrin synthesis in CEA is premature and can be detected in the cells immediately above the basal layer in stratified colonies, whereas filaggrin is not usually expressed. After transplantation, premature expres-
sion of involucrin in the immediate suprabasal layers may persist for several weeks to months, but normalization of filaggrin expression occurs rapidly after grafting and coincides with granular layer differentiation. Keratinocyte hyperproliferation is apparent by the marked increase in the number of cycling cells within the basal layer. Approximately one half to one third of all basal cells express Ki67, a nuclear antigen expressed by actively cycling cells, in the early postgrafting period (Fig. 6). Normalization of Ki67 expression (5–10% of basal cells express Ki67 in normal skin) requires several months. In contrast, expression of "hyperproliferative keratins" (i.e., cytokeratins 6 and 16) (Fig. 7) may persist for much longer than actual hyperproliferation as judged by increased cell cycling and may still be seen at one year or more postgrafting. Normalization of hyperproliferation markers appears to occur sooner in sole-derived CEA than in CEA derived from other body sites.

**Permanent Attachment to the Wound Bed and Basement Membrane Zone Formation**

Permanent attachment of the grafts to the connective tissue of the underlying wound bed requires de novo formation of basal lamina, mature hemidesmosomes and anchoring fibrils. None of these structures appear at the attachment face of the CEA at the time of grafting. Primitive hemidesmosomes and a discontinuous lamina densa are seen at the attachment face of the cell sheets in vitro, but they disappear with Dispase detachment of the CEA. By one week postgrafting, newly forming hemidesmosomes, basal lamina and anchoring fibrils appear together in discontinuous foci along the attachment face of the regenerating epidermis. By 3–4 weeks postgrafting, the basal lamina reaches confluency, hemidesmosomes are mature and show tonofilament insertion, and anchoring fibrils become thick enough to manifest their characteristic periodicity (Fig. 8). Until the basement membrane zone (BMZ) has matured to this point, CEA are easily dislodged from the wound bed and clinical protocols must allow for careful handling of the grafted sites. Completion of the BMZ corresponds
clinically to firm attachment to the wound bed.

The anchoring fibrils of epidermis regenerated from CEA are biochemically normal but structurally immature for 6–12 months postgrafting. They are sparse and thin compared to those of normal skin but are identical in appearance to the reforming anchoring fibrils in re-epithelialized interstices of MSTA on the same patients. From 1 week postgrafting, the pattern of ultrastructural immunolocalization of type VII collagen, their major structural component, is normal. Anchoring fibrils increase in density, thickness and architectural complexity for 6–12 months and ultimately become morphologically and morphometrically indistinguishable from those of age- and site-matched normal skin (Fig. 9).

Observations on anchoring fibril regeneration in epidermis derived from CEA have suggested that these structures are epithelial in origin. Ultrastructurally, a close spacial relationship between newly forming hemidesmosomes, basal lamina and anchoring fibrils is seen along the attachment face of CEA. In contrast, no specific spacial relationship between anchoring fibrils and wound bed fibroblasts is apparent. Furthermore, immunohistochemical studies demonstrate that type VII procollagen is expressed by the basal keratinocytes of transplanted CEA. The issue of anchoring fibril origin was clarified by transplanting cloned human CEA (pure keratinocyte sheets devoid of human fibroblasts) to nondermal connective tissue in nude mice. Newly formed anchoring fibrils at the human-mouse epithelial-stromal junction label strongly by ultrastructural immunolocalization using an antibody specific for human type VII collagen. These findings confirm the hypothesis that anchoring fibrils are primarily epithelial in origin and even demonstrate that anchoring fibril formation by human keratinocytes does not require the presence of human fibroblasts.

Epidermal Site-Specific Differentiation of CEA

Reexpression of site specific epidermal differentiation after transplantation of CEA has been demonstrated in CEA derived from sole skin transplanted to various diverse body sites. Sole skin has a highly distinctive phenotype and expresses a unique cytokeratin (CK9) permitting definite identification by objective criteria. Sole-derived CEA reexpress their native epithelial phenotype within a few weeks postgrafting and maintain that phenotype long-term (4-year follow-up). Although evident by histological criteria alone, reexpression of sole-specific phenotype is confirmed by immunohistochemistry and immunoblotting using a monospecific antibody to CK9 (generous gift of Dr. Werner Franke, Heidelberg, Germany). These findings indicate that in a permissive environment epidermal cells manifest an intrinsic capacity for site-specific differentiation. This phenomenon could be used clinically to the patients'
advantage. In bed-ridden patients, for example, buttocK or scapulae could be grafted with CEA expressing a phenotype specialized for weight-bearing.

Dendritic Cell Repopulation

All three types of epidermal dendritic cells (i.e., Langerhans cells, melanocytes, and Merkel cells) repopulate CEA during the first postgrafting year.

(1) Langerhans Cells (LC): Cutaneous LC are bone marrow derived elements that have the ability to induce antigen-specific T cell activation and are believed to participate in cutaneous immune responses as antigen-presenting cells. Although LC can persist in diminished numbers in organ cultures of human skin, they do not survive in cell culture and are absent from second passage CEA. However, immunohistochemical studies using anti-T6 antibody demonstrate rapid repopulation of CEA by LC after transplantation, undoubtedly due to result of transmigration from underlying connective tissues, lymphatics, or blood vessels. CEA of different body-site origins appear to be repopulated at different rates. By 2–3 weeks LC densities of sole-derived CEA are comparable to those of native sole skin, but in cultured grafts of axilla- and groin-skin origin a normal density of LC is not seen before 6–10 months. From 1–2 years postgrafting, LC densities in CEA are often increased significantly: 2–3 fold greater than normal in grafts of groin or axilla skin origin, and 4–6 fold above normal in sole-derived grafts. LC densities in reepithelialized interstices of MSTA are even higher, typically 3–7 fold greater than normal as early as 8 months after grafting. These results demonstrate that LC readily repopulate skin regenerated from CEA and are present in normal densities over the first 1–2 postgrafting years. Over time, LC densities in CEA exceed those of normal site-matched skin controls but are always greater than those observed in MSTA of comparable postgrafting age.

(2) Melanocytes: Melanocyte growth is supported by the culture conditions under which CEA are produced. Thus, melanocytes are present in CEA at the time of grafting. However, it may take weeks or months for functional epidermal melanin units (melanin transfer from melanocytes to surrounding basal keratinocytes) to develop and for CEA to become normally pigmented. When repigmentation occurs, the normal racial pattern of melanin distribution within the epidermis is reestablished. Freezing of disaggregated epidermal cells prior to or between passages in vitro decreases the number of surviving melanocytes [unpublished data] in CEA, and thus undoubtedly adversely affects repigmentation of CEA produced from frozen cells. In freshly passaged keratinocyte cultures, melanin transfer begins in vitro.

(3) Merkel Cells: Merkel (cutaneous neuroendocrine) cells are localized by immunohistochemistry using monoclonal antibodies to cytokeratin no.18 and/or neuron-specific enolase and confirmed by electron microscopy. They are absent from CEA prior to transplantation but appear in epidermis regenerated from CEA as early as 3 weeks postgrafting. This de novo differentiation documented in epidermis derived from CEA represents the first observation of Merkel cell differentiation in post-natal life in humans. Merkel cell differentiation appears to be independent of neural induction and to be related to epithelial site specificity since it has only been observed in CEA of sole-skin origin. These results indicate that Merkel cells do not originate from the neural crest as previously thought but rather derive from neuroendocrine differentiation of epithelial stem cells. The epithelial origin of the neuroendocrine system of the skin parallels that of the gastrointestinal tract and tracheobronchial tree in which the APUD ("amine precursor uptake and decarboxylation") neuroendocrine cells have also been shown to derive from epithelium.

Wound Healing in the Connective Tissue Subjacent to CEA

The wound bed subjacent to CEA is initially composed of granulation tissue that matures to normal scar within 6–8 weeks (Fig. 10). Through the first 6–12 months postgrafting, connective tissue wound healing in biopsies of CEA resembles that in MSTA intersticite controls. Thereafter, however, CEA develop rete
Fig. 10 Biopsy of CEA at 6 Months Postgrafting, Elastic-Tissue-Trichrome Stain

Fig. 11 Biopsy of CEA at 1 Year Postgrafting, Hematoxylin and Eosin Stain

Fig. 12 Biopsy of CEA at 5 Years Postgrafting, Elastic-Tissue-Trichrome Stain

Fig. 13 Biopsy of CEA at 5 Years Postgrafting, Hematoxylin and Eosin Stain

Fig. 14 Biopsy of MSTA Interstice Control at 5 Years Postgrafting, Hematoxylin and Eosin Stain

Fig. 15 Biopsy of MSTA Interstice Control at 5 Years Postgrafting, Elastic-Tissue-Trichrome Stain
ridges and a histologically distinctive neodermis\(^2\) . Stromal and vascular remodeling results in a bilayered collagen distribution pattern reproducing a papillary/reticular dermal structure and a superficial dermal capillary arcade that interdigitates with rete ridges (Fig. 11). At this stage (partial dermal regeneration), however, the neodermis lacks elastin. After 5 years, elastin regeneration is observed in the neodermis (complete dermal regeneration) (Fig. 12). Normal epidermal differentiation is maintained long-term with no indications of premature aging (atrophy) or cytological atypia (Fig. 13). The epidermis of MSTA interstice controls fails to develop rete ridges (Fig. 14), and the connective tissue stroma has the appearance of an old scar lacking in elastin, bilayered collagen distribution, and ordered vascular architecture (Fig. 15).

Biopsies of CEA at 3–8 years postgrafting have shown complete dermal regeneration subjacent to CEA in at least one biopsy from every patient at 5 years or greater postgrafting, whereas controls of age-matched MSTA interstices in these patients show only remodeled scar tissue. At 3–5 years postgrafting, partial dermal regeneration is seen in every case. These long-term results confirm that CEA produce a stable normal epidermis and are capable of inducing regeneration of dermis from wound bed connective tissue.

**Graft Reinnervation**

Immunohistochemical localization of neurofilament (NF) subunits (NF200, NF160, NF68), nerve growth factor receptor (NGFR) and tyrosine hydroxylase (sympathetic autonomic nerves) has shown that reinnervation patterns in CEA and controls are similar to one another on long-term follow-up but that a normal cutaneous innervation pattern is not reestablished. Perivascular nerves appear in the superficial wound bed beneath CEA or MSTA interstices within 4–8 weeks postgrafting and persist for years. However, free superficial dermal and intraepithelial nerve endings, as seen in normal skin, do not regenerate, and in the transplanted dermis of MSTA skin bridges, they quickly degenerate.

**Physiological Function in Skin Regenerated from CEA**

(1) **Sensory Perception:** In order to obtain objective neurophysiological data on sensory function in CEA and MSTA, we have performed quantitative sensory testing at 3 years postgrafting in an 11-year-old male who sustained 95% body burns (85% full-thickness) and was autografted with CEA and MSTA on mirror-image sites on the upper extremities. Large (thick) fiber sensory function was tested by determination of vibration thresholds using a Vibrometer\(^3\) . Small (fine) fiber sensory function was tested by determining temperature thresholds using a Thermostat\(^3\) . Results were compared to normative data from uninjured skin on the hands. By this analysis, thick fiber sensory function (vibratory sensation) is found to be fully reconstituted in both CEA and MSTA within 3 years postgrafting. Fine fiber sensory function (temperature sensation) remains subnormal in both graft types within this time frame. These observations coincide with our immunohistochemical study on graft reinnervation as outlined above (i.e., comparable patterns of sensory fiber ingrowth over time, but no full restoration of the normal skin pattern). The thermal sensory perception in CEA derived from groin skin is increased compared to MSTA, but in CEA derived from sole skin, it is decreased. This suggests that fine fiber sensory reinnervation in skin regenerated from CEA of various body-site origins may ultimately differ. These data, along with our evidence that CEA reexpress their original body site-specific differentiation after transplantation, further suggest that selection of the donor site for CEA production has important clinical implications for long-term graft performance.

(2) **Microcirculatory Function:** CEA, MSTA and healed partial-thickness wounds (HPTW) have been found to develop comparable microcirculatory function as measured by perfusion analysis on long-term follow-up. Using a laser-Doppler perfusion monitor (helium neon laser: wave length 632.5 nm; penetration...
0.7–1.5 mm), blood flow measurements were taken randomly and blindly over arms, legs, face and thorax on a 13-year-old male grafted with CEA over more than 50% of his body surface following a 98% burn 8 years ago. All perfusion measurements were made in a constant temperature environment (23°C), since the cutaneous blood supply is known to be sensitive to environmental temperature changes. No statistically significant variances were found among values between groups after comparing HPTW vs. CEA, HPTW vs. MSTA, and CEA vs. MSTA. Thus, our data indicate that by 8 years postgrafting, both CEA and MSTA have comparable dermal microcirculatory function and that their dermal perfusion is comparable to healed partial-thickness wounds in the same patient.

**New Perspectives**

Currently, one of the most significant problems in the clinical use of CEA is the variable rate of successful engraftment (graft "take"). When grafted to wound bed granulation tissue, the average take-rate of CEA is 55–60%, as reported in the 3 large patient series extant in the literature15, 24, 25 including our own experience. New clinical data from 16 patients in 7 burn centers in the U.S.A. using commercially produced CEA (BioSurface Technology, Inc., Cambridge, MA) have indicated that transplantation of CEA to the dermal remnant of engrafted, partially-excised homograft in a two-step procedure, a method introduced by Cuono and his coworkers39, improves the rate of successful CEA engraftment significantly. When grafted to allodermis, the rate of successful engraftment averages 85% (range 60–100%), a take-rate comparable to MSTA40. Thus, this approach to CEA grafting has proven both practical (homograft being the best temporary wound cover in any case) and successful in improving the clinical performance of CEA. In addition, our analysis of biopsies received from patients treated by this method indicates that many biological events in the process of skin regeneration, including rete ridge formation and normalization of keratin programs, are accelerated when CEA are grafted to allodermis (Fig. 16) rather than

**Fig. 16 Biopsy of CEA Transplanted to De-epidermized Homograft at 7 Months Postgrafting**

**Fig. 17 Biopsy of CEA Transplanted to Granulation Tissue at 7 Months Postgrafting**

**Conclusion**

CEA represent a lifesaving technique for providing rapid, permanent wound coverage in massive burns. They regenerate a normal stable epidermis and modulate healing in the subjacent connective tissue of the wound bed leading to dermal regeneration. The skin that regenerates from CEA is physiologically comparable to standard MSTA and more closely resembles normal skin histopathologically than MSTA interstice controls on long-term follow-up. Transplantation of CEA to wound beds prepared with deepidermized homograft dermis according to the method of Cuono et al.39 accelerates many of the key events in wound repair and skin regeneration as compared to CEA transplanted to wound bed granulation tissue.
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