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Cultured Skin Substitutes: A Review

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ABSTRACT

Skin substitutes composed of cultured cells and biopolymers provide alternative materials for study of skin biology and pathology, treatment of skin wounds, safety testing of consumer products, and therapeutic delivery of gene products. Most frequently, substitutes for epidermis consist of cultured keratinocytes and dermal substitutes consist of resorbable biopolymers populated with cultured fibroblasts. Preclinical models characterize cellular morphogenesis, antigen expression, and barrier properties *in vitro*, and recovery of tissue function after grafting. Clinical considerations include time required to prepare cultured autografts, time required for graft vascularization, management of microbial contamination in wounds, mechanical fragility of cultured grafts, and high cost. Safety in graft preparation generally requires the use of materials and procedures that comply with standards for quality assurance. Efficacy of engineered skin substitutes has been evaluated predominantly by subjective criteria, but evaluation may become more objective and quantitative by application of noninvasive biophysical instrumentation. Future directions with engineered skin substitutes are expected to include gene therapy by addition or deletion of selected gene products and establishment of international standards for fabrication and assessment of engineered skin.

OBJECTIVES OF CULTURED SKIN SUBSTITUTES

BIOLOGIC SUBSTITUTES OF human skin have several prospective applications including, but not limited to, the following:

1. Models of skin biology and pathology.
2. Treatment and closure of skin wounds.
3. Alternatives to animals for safety testing of consumer products.
4. Delivery and expression of transfected genes.

Each of these prospective applications has distinct requirements for validation of skin substitutes.[1-4] These categories of applications represent, respectively, the fields of investigative dermatology, surgery, toxicology, and gene therapy. This article reviews factors and considerations with respect to treatment and closure of skin wounds. However, all applications have a common set of requirements for anatomic and physio-

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logic fidelity of skin substitutes to native human skin. It is important to recognize that no engineered skin substitute has yet duplicated *in vitro* all of the structures and functions of native human tissue.

For use in wound closure, the first definitive requirement is reestablishment of the epidermal barrier to fluid loss and microorganisms.[5] In full-thickness skin loss, replacement of both the epidermis and dermis has gained consensus.[6] Replacement of these tissue compartments must also minimize scar and restore acceptable function and cosmesis.[7] Perhaps the most important objective for skin substitutes in wound closure is to reduce or eliminate the donor site for skin grafts.[8] If this objective is satisfied, hypothetically recovery time is shortened and operative procedures are reduced. For patients who are poor candidates for surgery, elimination of donor sites by use of skin substitutes containing allogeneic cells may reduce risks associated with surgery.

Conventional grafting expands donor skin by about 1:4. In contrast, rapid growth of cells *in vitro* allows coverage of culture surfaces by more than 1,000 times the area of the skin biopsy.[9,10] Growth rates of keratinocytes and fibroblasts in culture have been quantified at approximately one population doubling per day, and colony-forming efficiency of keratinocytes in primary culture is 1–10% of cells isolated from tissue.[11–13] Because cell growth is an exponential function (2^n, where n = population doublings), cell numbers in culture increase by an approximate factor of 1×10^3 in 10 days, and 1×10^6 in 20 days. Even with low efficiency of graft preparation, coverage of the total body with autologous skin cells may be accomplished from a biopsy specimen smaller in area than the palm of the patient's hand, or about 1% of the body surface. Hypothetically, if the patient's life can be supported long enough for culture of skin cells (about 3 weeks) and for healing of transplanted cells (1–2 weeks), mortality from catastrophic skin loss injuries may be reduced. The increase in availability of skin grafts for wound closure based on exponential cell growth is the fundamental medical advantage that drives the research and development of cultured skin substitutes. However, the virtually unlimited availability of cultured cells does not solely satisfy the requirements for safe and effective wound closure with skin substitutes.

DESIGN CONSIDERATIONS

Design of cultured skin substitutes must address the following categories of considerations:

1. Restoration of skin anatomy and physiologic function.
2. Genotype of transplanted cells.
3. Biocompatibility of polymeric materials.
4. Cost and complexity of fabrication.
5. Storage or banking of components.

Basic design considerations for an "artificial skin" have been described to include control of infection, fluid loss, contracture, and scarring.[14] These requirements are part of a larger set of requirements for temporary and permanent skin substitutes that include rapid adherence and vascularization, mechanical stability and durability, and cost-effectiveness.[15] Restoration of skin anatomy should include not only epidermis and dermis, but also skin pigmentation, nerve, vascular plexus, and adnexa (glands, follicles). Although reasonable analogues of epidermal skin have been developed[16–19] and skin pigmentation has been restored clinically by transplantation of cultured melanocytes,[20] most skin substitutes do not contain biopolymers with native molecular structure. Studies to include nerve, blood vessels, and glands remain in preliminary stages of experimentation.[21,22]

Cells for preparation of skin substitutes may be propagated from autologous or allogeneic tissues. Autologous cells can be permanent,[23] but they require 3–4 weeks to propagate in sufficient numbers to transplant therapeutically. Conversely, allogeneic cells have been shown to persist only temporarily but provide virtually unlimited availability.[24] In general, these cell sources serve, respectively, acute wounds (i.e., burns, trauma), and chronic wounds (cutaneous ulcers).[25] An exciting potential for improved utilization of autologous cells is preparation of cultured skin from chimeric cell populations. Although this approach has been demonstrated in preclinical studies,[26,27] clinical studies with chimeric grafts are not yet reported. However, feasibility for clinical use of chimeric grafts is supported strongly by the well-established use of combined autologous and allogeneic skin grafts[28] if donor autograft has limited availability.

Biopolymers for cultured skin substitutes may be tissue derived or synthetic.[29–32] Collagen is commonly used in medical devices as an implant or a coating onto which cells can attach and migrate during healing. Medical grades of collagen range in nativity from low, as in gelatin sponges,[33] to high, as in decellularized cadaver dermis.[34] In general, nativity of collagen structure delays degradation. Other natural biopolymers of increasing importance are hyaluronic acid,[35] which has been associated with scarless wound healing in the fetus,[36] and fibrin.[37] By comparison, synthetic polymers (i.e., polylactic acid [PLA] and polygalactic acid [PGA]) have become standard for certain medical applications including suturing and repair of the abdominal wall. Fabric made from PLA/PGA has been studied as a vehicle for delivery of skin cells but was associated with extrusion of the polymer from the wound.[38] Microbial contamination of PGA/PLA grafts has been reported to be lower than with collagenous implants,[39] but cell survival of transplanted cells is also reduced. These results suggest that release of acidic monomers during hydrolytic degradation of the polymer may generate a sufficiently acidic pH to be cytotoxic.

As engineered tissues become more sophisticated, cost and complexity of fabrication also increase. Although these factors are sometimes discounted, complexity of fabrication should be considered initially, if engineered tissues are to be made practical. Fibroblast-populated collagen gels with cultured keratinocytes[19] have been demonstrated to generate analogues of skin with good histologic fidelity to skin. Although allogeneic forms of this model have been used to treat chronic wounds, it has not been practical for preparation with autologous cells because of the long maturation periods during fabrication.

Essential to availability of tissue-engineered skin is technology for storage or banking of individual and composite materials. In general, hydrated materials (cells, biopolymers) must be cryopreserved for long-term storage[40] or dehydrated chemically or physically[34] to reduce rates of decomposition. Mammalian cells do not survive drying, but acellular materials are most often stored dry after desiccation by freeze-drying.[34,41,42] Each of these storage techniques allows accumulation of materials for subsequent preparations or applications. Many principles and standards of storage of tissue and tissue-derived materials have been established by the practice of tissue banking and organ procurement for medical transplantation.[43,44] Although specific procedures for tissue storage remain variable from one institution to another, most of the principles and standards can be adapted directly for engineered tissues.

PRECLINICAL MODELS

Composition and performance of skin substitutes must be evaluated before initiation of clinical studies. Similar to other experimental therapies, cultured skin substitutes may be validated for function by evaluation *in vitro* and in animals. Factors to be evaluated include, but are not limited to, the following:

1. Regulation of cellular proliferation.
2. Morphogenesis of cells into skin analogues.
3. Histogenesis of skin analogues into functional skin.
4. Biochemical and biophysical assessment.

With few exceptions,[45] techniques for transplantation of parenchymal cells involve growth *in vitro* to expand exponentially cell numbers.[9,12] Generally, this requires selective growth of a cell type(s) of interest. For skin, epidermal keratinocytes are definitive for wound closure. Combination of cultured keratinocytes with an acellular biopolymer matrix[16] probably constitutes the minimum essential requirement for definitive restoration of skin function. Fibroblasts have been incorporated into certain models[46,47] to promote reestablishment of epithelial-mesenchymal interactions that are recognized[48,49] but remain poorly understood. Addition of epidermal melanocytes to cultured skin *in vitro* has also been reported.[50,51] As cellular complexity of engineered skin increases, formulation of media also becomes more complex. Although cells at high density are subject to the same requirements for essential nutrients as low-density cultures,[52] both rates of cellular metabolism and requirements for exogenous growth factors decrease generally as skin analogues form.[53] In cultured skin models, exposure of the epithelium to air also promotes formation of epidermal barrier,[54] which may be considered "mophogenesis." Epidermal barrier has been measured quantitatively by transepidermal water loss,[55–57] percutaneous absorption of chemicals and radio-isotopes,[56,58]

surface electrical capacitance,[59,60] biochemical analysis of barrier-specific lipids,[58,61] and x-ray diffraction of stratum corneum.[62] Cultured keratinocyte sheets[10,23] cannot be evaluated for barrier properties *in vitro* because they are prepared in submerged culture. However, epidermal barrier function has been demonstrated transiently and at only 75–90% of values for native skin,[55,56,63] and no other skin functions are recovered. Deficiencies in epidermal barrier have been associated with excessive accumulation of triglycerides in keratinocytes and very low levels of stratum corneum lipids.[61,64] These deficiencies are consistent with a hyperproliferative cellular phenotype. In most models, mechanical properties of skin substitutes *in vitro* are less than 10% of native skin. But, use of acellular dermal matrix[34,65,66] or fabrics of absorbable polymers (i.e., PLA/PGA)[38,39] provides some elasticity and strength similar to native skin. Melanocytes have been shown to reside passively in skin substitutes,[47,50,67] and measurement *in vitro* of pigment expression with the chromameter[68] consistently shows less color than in the donor skin. Vascular plexus, nerves, glands, follicles, and immune effector cells are absent. Furthermore, the perishable nature of skin substitutes *in vitro* confirms the deficiencies of current culture systems.

After grafting in animal models,[47,69] pigmentation varies from partial to complete[51,70] and adnexa do not reform. However, transplantation of cultured skin regenerates functional and stable barrier and repairs fibrovascular tissue. Because most models of skin substitutes are prepared with human cells and are xenografts, athymic mice have been used for long-term studies by several laboratories.[47,59,69,70] Grafting of autologous or allogeneic keratinocytes to immunocompetent pigs[71] has been performed for short-term studies because pig skin is considered most similar to human skin in anatomy and physiology. Stable restoration of barrier function after grafting may be termed "histogenesis" because a definitive function of skin tissue is regained. Quantitative endpoints for these studies have included, but are not limited to, wound area,[51,72] ordinal scoring of human cells markers,[65] and expression of specific proteins, mRNAs, and lipids.[67,73,74] Particular attention has been given to detection and quantification of factors for angiogenesis and proliferation (i.e., basic fibroblast growth factor, transforming growth factors α and β, platelet-derived growth factor, and insulin-like growth factors), inflammatory mediators (i.e., interleukins and arachidonic acid metabolites), and extracellular matrix components (i.e., collagens, polysaccharides, laminin, and kalinin) by transplanted cells.[75–78] Together, the qualitative and quantitative proportions of cellular and extracellular factors define tissue integrity. Findings from these studies demonstrate consistently the following: that epithelium of skin substitutes is hyperproliferative and incompletely keratinized *in vitro*; that cell proliferation and epidermal barrier normalize after grafting; and that restoration of mechanical strength requires a dermal substitute. However, because the functions of glands, follicles, and nerve are not fully recovered, true "organogenesis" of skin has not yet been accomplished. Even grafts of native split-thickness skin do not contain adnexa because glands and follicles are generated only during development, not in wound healing. Therefore contemporary models of engineered skin are based on duplication *in vitro* of wound healing physiology, not developmental physiology.

Assessments of efficacy of cultured skin substitutes in preclinical models have certain strengths and limitations. Rates of engraftment and wound area can be determined with reasonable validity. But because rodents do not scar extensively, formation of keloid or hypertrophic scar cannot be evaluated well. However, recovery of skin function can be evaluated with biophysical instrumentation,[79] and molecular analysis can be performed in comparison to native human skin. Normalization of function (i.e., epidermal barrier) does not necessarily reflect normal metabolism (i.e., barrier lipid profiles).[80] For these analyses preclinical models are very useful, but they are influenced by host metabolism that may introduce artifact in comparison to clinical performance of grafts.

CLINICAL CONSIDERATIONS

Anatomic and physiologic deficiencies in epidermal barrier and vascular supply confer on skin substitutes practical limitations that must be managed to accomplish efficacy of wound closure. Among these limitations are the following:

1. Mechanical fragility.
2. Susceptibility to microbial contamination.

3. Decreased rates of engraftment compared to autograft.
4. Increased time to completion of healing.
5. Very high cost.

Early reports with clinical application of keratinocyte sheets proved the life-saving potential of cultured skin substitutes.[23,81] These studies also identified multiple limitations to their routine use according to procedures for split-thickness skin. If grafted onto subcutaneous fat after excision of full-thickness burns, keratinocyte sheets attach poorly and are destroyed by mechanical shear and microbial contamination. To improve engraftment, proponents recommended during the 1980s excision to muscle fascia that resulted in removal of viable tissue and disfigurement. It was also noted that healed epithelium subsequently ulcerated and often required regrafting. These results increased rather than decreased time to completion of healing. In addition, the cost of preparation of keratinocyte sheets for large burns is extremely high. Later studies combined keratinocyte sheets with cadaveric allograft from which the allogeneic epidermis had been surgically removed.[82] This approach has gained more common use, and is reported to have fewer complications.[83] However, it requires availability of viable cadaveric allograft skin.

Combination of epidermal cells with dermal substitutes *in vitro* has been shown to virtually eliminate epidermal blistering after engraftment of transplanted keratinocytes.[84] More rapid formation of basement membrane and anchoring fibrils[85,86] in dermal-epidermal models may result from initiation of cell-substrate attachments *in vitro* and elimination of enzymatic release of cell sheets immediately before grafting. An important limitation of composite skin substitutes is lack of vascular supply in the dermal substitute. This limitation extends time for reperfusion, ischemia, and nutrient deprivation to transplanted cells that reduce rates of engraftment. Cell survival and engraftment may be improved significantly by irrigation of grafts with solutions of nutrients[87] and noncytotoxic antimicrobial agents.[88–90] This approach optimizes the wound environment to promote survival of transplanted cells and manage microbial contamination until vascularization is complete.

The high cost of cultured skin substitutes remains a practical factor for clinical use. Estimates of cost of keratinocyte sheets range from $1,000 to $13,000 per percentage of body surface area covered.[91,92] Those costs can approximately double if a dermal substitute is also used.[66,93] Therefore costs can become limiting for treatment of large (60–90% of total body surface area) burns with cultured skin substitutes. At present, no studies have convincingly demonstrated a savings of total hospitalization costs by use of cultured skin substitutes of any kind. For contemporary treatment of burns, cultured skin substitutes remain an important adjunct to conventional skin grafting[94] but are not a primary modality of wound closure except in extreme cases.

REGULATION AND ASSESSMENT

With appropriate management of clinical limitations, wound closure with cultured skin substitutes has been demonstrated for acute and chronic wounds.[2,24,95,96] Cultured skin substitutes are part of a spectrum of materials extending from cadaveric skin allograft to tissue-derived materials (keratinocyte sheets, dermal matrix), to specific combinations of cells and purified polymers. Because some kinds of skin substitutes, including cultured keratinocyte sheets, were considered initially a form of tissue banking, requirements for demonstration of efficacy were virtually absent.[97] Conversely, cultured skin substitutes consisting of cultured cells attached to biodegradable polymers are currently regulated in the United States as class III (significant risk) medical devices with rigorous requirements for demonstration of safety and efficacy, and compliance with good manufacturing practices (GMPs).

Regulatory complexities among these materials have been recognized by the U.S. Food and Drug Administration (FDA) and the European Committee for Standardization (CEN). The FDA has responded by assembly of an Inter-Center Focus Group on Wound Healing to explore whether uniform standards for evaluation of safety and efficacy can be established for wound treatment materials.[98] This ambitious goal may provide, at minimum, a set of reference endpoints and, at maximum, an absolute set of parameters and methods for evaluation of experimental therapies including tissue-engineered skin. Similarly, The CEN has established three directives pertaining to medical devices: the Medical Devices Directive, the Active

Implantable Medical Devices Directive, and the *In Vitro* Diagnostic Medical Devices Directive.[99] The CEN adopted its directives in the 1990s as part of the process of harmonization within the emerging European Community. Many parallels exist between the FDA and CEN standards, for example, in classification of medical devices. As economic globalization proceeds, resources will be conserved if regulatory standards can be harmonized concurrently. The International Standards Organization has established regulations for control of sterility in product manufacture that overlap with standards for GMPs.[100,101] Because the underlying factors for medical safety and efficacy supersede national citizenship, progressive globalization of standards for medical devices that include cultured cells may prospectively be addressed by the World Health Organization or similar affiliation of international experts. Current standards in the United States recognize generally that wound closure (reepithelialization) is an acceptable endpoint of wound healing with cultured skin substitutes. However, a distinction can readily be made between percentage of engraftment, and percentage of coverage of the body surface because in burns, percentage of engraftment of cultured skin is inversely related to magnitude of injury.[102] Standardization of these kinds of endpoints and distinctions in assessment will benefit all patients who are treated with cultured skin substitutes.

Particular attention has been given to methods for noninvasive evaluations of skin based on data from biophysical instrumentation.[79] The importance of noninvasive assessment is emphasized by a multiplicity of instrumentation that has been developed independently by investigators from around the world. Advantages of this approach include reduction of subjectivity in data collection[103,104] and availability of instrumentation to any investigative center.

Prospective measurements from skin with available instrumentation may include the following:

1. Viscoelastic (mechanical) properties.[105–107]
2. Epidermal barrier (surface hydration).[59,60]
3. Blood flow.[108]
4. Surface contour.[109]
5. Pigmentation and erythema.[110,111]

However, the selection and development of standards will require extensive validation and consideration of practical limitations to recommended uses of candidate instruments. For example, both the evaporimeter and the dermal phase meter measure epidermal barrier.[112] However, environmental requirements (ambient humidity, air currents) for operation of the evaporimeter make it relatively impractical for use with intensive care patients. In contrast, the dermal phase meter is not subject to these limitations and serves efficiently and conveniently in all human subjects.[60,113] Skin elasticity has also been measured with the cutometer to evaluate burns grafted with meshed skin graft or cultured keratinocyte sheets over dermis.[107]

Although existing instruments have been calibrated and validated for individual endpoints in narrow populations, correlation among endpoints has received only exploratory attention.[114] Validation for broad-use individual or aggregate assessments will require the following:

1. Characterization of subject variables (age, race, gender).
2. Distinction of normal and pathologic skin.
3. Correlation of biophysical (surrogate) measurements with clinical outcome.
4. Multiparameter score for skin condition.
5. Development of consensus among clinicians.

Because native skin may be characterized by its individual biophysical characteristics, it may hypothetically be quantified as a composite score of multiple biophysical properties. If valid, that score should be a substitute for clinical examination. Electrocardiograms, electroencephalograms, magnetic resonance imaging, computerized axial tomography, and radiography are examples of noninvasive biophysical instruments that have provided diagnostic data beyond the perception and resolution of a clinical examination.

FUTURE DIRECTIONS

As anatomy and physiology of cultured skin substitutes improve, they will become more homologous to native skin graft. Improvement of skin substitutes will result from modifications of culture media, sub-

strates, and physical environment (i.e., humidity, mechanical tension, electrical properties) that have more fidelity to native skin. Better homology may be expected to reduce stringency for clinical use of cultured skin substitutes and accomplish the efficacy of skin autograft. After predictable efficacy is shown with autologous cells, successful models will become platforms for testing of chimeric grafts and genetically modified cells.[77,78,115,116] Gene therapy for treatment of local or systemic conditions is feasible with cultured skin substitutes.[4,77,115,117–119] For wound healing applications, transient expression of selected gene products may be best, whereas constitutive gene expression may be required for systemic deficiencies. Delivery of gene products systemically probably will require physiologic regulation to be efficacious.[120]

If engineering of skin and other tissues is to become a true science, members of its community must begin to think and act like engineers. Therefore, like other engineering disciplines, uniform standards for quantitative analysis of cultured skin substitutes must be established for evaluation of materials composition and performance. This goal will require interdisciplinary review and development of consensus. However, establishment of these engineering standards will provide a platform from which validation and introduction of skin and other tissue substitutes will be expedited. Although the term "tissue engineering" describes a relatively new interdisciplinary field, it has its roots many years past in the fields of cell culture and polymer chemistry.[121–123] If past progress in engineering of cultured skin substitutes is an indication of progress ahead, it is easy to predict the reduction of medical morbidity and mortality by use of culture skin substitutes in the future.

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