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# Fabrication, quality assurance, and assessment of cultured skin substitutes for treatment of skin wounds

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#### Abstract

Advances in treatment of skin wounds depend on demonstration of reduced morbidity or mortality either during or after hospitalization. Tissue engineering of skin grafts from cultured cells and biopolymers permits greater amounts of grafts from less donor tissue than conventional procedures. Autologous keratinocytes and fibroblasts isolated from epidermis and dermis of skin may be combined with collagen-based substrates to generate cultured skin substitutes (CSS) with epidermal and dermal components. By regulation of culture conditions, CSS form epidermal barrier and basement membrane, and release angiogenic factors that stimulate vascularization. Prototypes of CSS may be tested for safety and efficacy by grafting to athymic mice which do not reject human tissues. Clinical application of CSS requires establishment of quality assurance assessments, such as, epidermal barrier by measurement of surface hydration, and anatomy by standard histology. Medical benefits of tissue engineered skin for treatment of burns are evaluated quantitatively by the ratio of healed skin to donor skin, and qualitatively by the Vancouver Scar Scale. These benefits may also be extended to other medical conditions including chronic wounds and reconstructive surgery.

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## 1. Introduction

Cultured skin substitutes of multiple compositions have been designed and tested for treatment of skin wounds [1-3], or as alternatives to animal testing for toxicology and pharmacology [4]. For wound treatment, general approaches have included cells only, polymers only, or combinations of cells and polymers. Most models for treatment of skin wounds were prepared in vitro, but some utilized intra-operative procedures with incubation of cell populations in vivo [5] to enhance tissue repair.

The central hypothesis of the cultured skin substitute described here was whether reproduction in vitro of the anatomy and physiology of split-thickness human skin provided equal or better qualitative outcome with greater availability. This hypothesis was been tested by design, specification and fabrication of skin substitutes with dermal and epidermal components to provide the essential properties for wound healing. Although natural skin provides

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many anatomic structures and physiologic functions for the human body, the essential properties for wound closure may be referred to as the three B's: epidermal barrier to close the wound, basement membrane to bond the epidermis to the body, and blood supply from connective tissue in the wound. To accomplish these essential requirements, an approach of deconstruction and reconstruction of the skin has been studied [6]. The general processes for reconstruction of skin in vitro include: (1) cytogenesis to provide cells to repair the damaged tissue; and (2) morphogenesis to organize the cells together with a polymer to generate an analog of skin that can be handled and grafted to wounds. This approach has generated cultured skin substitutes containing human keratinocytes and fibroblasts attached to collagen-glycosaminoglycan substrates for clinical treatment of burns, chronic wounds and giant nevi [3]. However, melanocytes and microvascular endothelial cells have been added in preclinical studies to restore pigmentation and promote vascularization [7,8]. Cells in CSS have also been modified genetically to deliver specific proteins into the wound from the graft [9,10]. These features have contributed importantly to the clinical success of this model, and to prototypes of engineered skin with specific properties for specific purposes.

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Fig. 1. Diagram of the general process for tissue engineering of cultured skin substitutes. A small biopsy of split-thickness skin is separated with enzymes to isolate epidermal keratinocytes and dermal fibroblasts for culture. Epidermal melanocytes and dermal microvascular endothelial cells may also be isolated and inoculated into selective culture. Skin cells are grown into large populations, and inoculated onto biopolymer substrates prepared from collagen and glycosaminoglycan. Incubation at the air-liquid interface promotes epidermal differentiation and morphogenesis of the cells into an analog of human skin that may be transplanted as a graft.

## 2. Methods and results

## 2.1. General process

Fig. 1 shows the general process for deconstruction of skin for reconstruction of CSS. Selective cultivation of skin cells may be performed in conventional culture flasks, or in bioreactors to reduce requirements for labor and materials. Current studies with the Kerator bioreactor [11,12] are expected to begin a process of automation that will increase availability of CSS by reducing costs of fabrication. This first step in tissue engineering, cytogenesis, provides a large cell population with a high rate of proliferation that is needed to generate viable tissue.

#### 2.2. Specific process

Collagen–glycosaminoglycan (GAG) membranes are fabricated from bovine skin collagen and chondroitin-6-sulfate to generate biopolymer substrates with controlled thickness and pore diameter. Collagen is solubilized and co-precipitated by addition of chondroitin-6-sulfate at a controlled rate [13]. The mixture is homogenized, cast into sheets, frozen by submersion in a bath of 95% ethanol, and lyophilized overnight. The dry substrate is treated by thermal-dehydration in a vacuum oven at  $105 \,^{\circ}$ C for 24 h, packaged, sterilized by gamma irradiation, and stored for later use.

A biopsy of split-thickness skin ( $\sim 250-300 \,\mu\text{m}$  thick) is collected from a burn patient, usually during the first procedure for autografting. The area of the biopsy is estimated as  $\sim 1\%$  of the absolute value of the area (cm<sup>2</sup>) to be treated. The biopsy is dissociated with dispase to separate the epidermis and dermis from which keratinocytes and fibroblasts are isolated with typsin-EDTA and collagenase, respectively [14,15]. Selective cultures of keratinocytes and fibroblasts are grown to generate stocks of cells that are cryopreserved by controlled-rate freezing, and stored in liquid or gas-phase nitrogen at -196 °C. Subsequently, keratinocytes and fibroblasts are expanded in media for selective growth of each cell type [16,17]. After rehydration of sterile, dry collagen–GAG substrates, fibroblasts and keratinocytes are inoculated sequentially onto the substrates, and incubated with the epithelial surface in contact with the atmosphere [18]. After 10-14 days of incubation, CSS are ready for grafting to patients.



Fig. 2. Histology of a cultured skin substitute (CSS). The CSS consist of an epidermal component (E) that contains epidermal keratinocytes, attached by basement membrane proteins to a dermal component (D) that contains dermal fibroblasts attached to a collagen–glycosaminoglycan sponge. The CSS are similar to split-thickness skin grafts in anatomy and physiology. Scale bar represents 0.1 mm.

## 2.3. Quality assurance in vitro

The cells organize by morphogenesis into a tissue analog with expression of tissue-specific phenotypes to restore partially the structure and function of skin (Fig. 2). Epidermal barrier may be monitored by measurement of surface hydration with the Dermal Phase Meter (Fig. 3A) [19,20]. This measurement is used currently for quality assurance of CSS for clinical investigations. Surface hydration of CSS in vitro with optimal quality for grafting is not different from healthy skin of normal volunteers (Fig. 3B).

## 2.4. Preclinical grafting to athymic mice

After establishment of appropriate protocols for animal subjects, prototypes of CSS are tested for development of functional tissue ("histogenesis") by grafting to full-thickness skin wounds in athymic mice (Fig. 4). Persistence of cultured grafts is compared to grafts of natural skin from the recipient mouse (autograft), or from a human donor (xenograft). Performance of the cultured graft may be assessed by histology, persistence of human cells by labeling of HLA-ABC antigens, and by measurement of original wound area [21]. If the engineered graft compares favorably to the natural grafts, that prototype can be considered for treatment of clinical wounds after establishment of appropriate human subjects protocols.

## 2.5. Surgical and nursing procedures

Planning of clinical studies should consider carefully the standard practices for surgery and nursing care of natural skin grafts. For treatment of burns, CSS are delivered to the operating room with a covering of N-Terface, a non-adherent dressing that is stapled to the wound together with the graft. CSS are compared to split-thickness skin autograft (AG) in paired sites in each patient. CSS and AG are maintained in moist dressings for 5 days after surgery with a formulation of antimicrobial agents in a nutrient solution or in saline [22]. The antimicrobial solutions for AG consist of 5% (w/v) mafenide acetate, alternating with a mixture of polymyxin B and neomycin. The antimicrobial solutions for CSS must maintain antimicrobial activity against a broad spectrum of organisms, including Gram-negative



Fig. 3. Surface hydration as an index of epidermal barrier. (A) Measurement of a cultured skin substitute with the Nova Dermal Phase Meter 9003; (B) data from the Dermal Phase Meter that shows drying of the epidermal surface of CSS ( $\bullet$ ) to the same values as healthy skin from normal volunteers (dashed line).



Fig. 4. Healing of CSS on athymic mice. CSS are grafted to full-thickness surgical wounds in athymic mice with the panniculus carnosus preserved. (A) CSS depleted of melanocytes generate non-pigmented skin (inside dotted line). (B) CSS with added melanocytes generate pigmented skin on this albino animal.

and Gram-positive bacteria, and fungi; and, be non-cytoxic to transplanted cells. Previous studies from this laboratory have determined an effective and non-cytotoxic formulation consisting of polymyxin B, neomycin, mupirocin, ciprofloxacin and amphotericin B [23]. CSS grafts are irrigated with this solution for 5 days after grafting, and subsequently the remaining open areas are treated until closed with an ointment consisting of equal parts of Neosporin, Bactroban and Nystatin. After closure, healing grafts are treated with a moisturizing lotion or cream.

## 2.6. Assessment of outcome

For burns, two types of data sets are collected, quantitative and qualitative. Quantitative data require determination of the areas by computerized planimetry of: (a) the biopsy collected to initiate the cell cultures; and (b) the grafted wounds at post-operative days (POD) 14 and 28. The area of the biopsy is the amount of skin used to close the wounds. The area at POD 14 allows determination of the total area grafted, and the area closed to calculate the percentage engraftment. The area at POD 28 allows determination of the area closed by CSS compared to the area of the original biopsy to calculate the ratio of closed wound to donor skin. Qualitative data consist of comparisons of CSS and AG by the Vancouver Scale for Rating the Burn Scar [24,25]. This scale provides ordinal scoring of vascularity (red color), pigmentation (brown color), pliability, and scar height. This system is widely accepted in the community of burn care providers, but it is somewhat subjective. Skin condition may also be measured with non-invasive biophysical instruments which may include measures of skin color, texture, visco-elastic properties, pH, or temperature [26].

Fig. 5 shows the representative results from CSS or AG applied over excised, full-thickness burns. Data collected from burn patients after treatment with CSS or AG show that engraftment is very similar, wound closure per unit donor skin is much greater for CSS (Fig. 6), and qualitative outcome is not different between the two graft types [27]. Importantly, CSS are synergistic with the dermal substitute, Integra Artificial Skin [28]. These results are consistent with the replacement of a uniform layer of connective tis-



Fig. 5. Healing of CSS on a burn patient. CSS or split-thickness skin autograft (AG) grafted to excised, full-thickness burn injuries at post-operative days: (A) 28; (B) 69; and (C) 479. Scale in cm.



Fig. 6. Quantitative assessment of CSS and AG in treatment of burns. Tracing of healed and open areas after grafting shows: (A) engraftment of CSS at post-operative days 14 is compares favorably with AG; and (B) the ratio of closed area to donor area for CSS is >65, but for meshed autograft is  $\leq 4$  (P < 0.01). (Reprinted from Boyce et al., Ann. Surg. 235, 269–279, with permission.)



Fig. 7. Diagram of the general process of discovery, development and delivery of technology for advanced medical therapies. Discovery of new knowledge in basic studies leads to development of prototypes that may be protected with patents, and tested in preliminary clinical studies. Successful prototypes are licensed to commercial developers, tested in multi-center clinical studies, and manufactured for greater availability of advanced therapies.

sue by Integra, and the closure of the wound with CSS that provide epidermal barrier. Together these quantitative and qualitative advantages of CSS over AG provide an advanced alternative to harvesting of split-thickness skin.

## 3. Discussion

Engineering of CSS is part of the overall process of tissue engineering which contains three parts as shown in Fig. 7, referred to as the three D's: discovery, development and delivery. Until all three of these parts are completed, the standards of clinical care in the medical community do not change. Discovery of new knowledge results in the development of prototypes for testing in preclinical models to evaluate whether hypothetical advantages may exist for clinical care. After completion of successful experiments in animal models, initial clinical studies are conducted to determine whether any prospective benefit may be translated to patients. This begins the step of development of the engineered tissue, and if successful, initial studies may be expanded to demonstrate efficacy in a small patient population, usually at a single clinical site. If the experimental therapy is safe and efficacious, then it may be tested in multi-center studies to provide independent validation of the clinical benefits from the initial studies. Usually, a commercial partner will get involved as the therapy moves from the step of development to the step of delivery. Ultimate commercialization requires establishment of standardized manufacturing practices that assure patient safety and product quality.

Performance of clinical studies in the USA requires establishment with the US FDA of an investigative protocol which includes standards for quality assurance of an experimental therapy. For this model of CSS, histology, surface hydration by the Dermal Phase Meter, and sterility testing are the main criteria by which quality to the patient is assured. Hypothetically, these standards for quality assurance could be used by any investigators of skin substitutes.

The American Society for Testing and Materials (ASTM) in the United States has provided standards for medical devices under its Committee F04 for several decades. In 1997, Committee F04 established Division IV to develop standards for Tissue Engineered Medical Products (TEMPs) [29]. The membership of ASTM F04, Div IV, is open to participants from academics, industry and government. Standards developed by ASTM are often referred to by the US FDA for regulatory approval of investigative therapies. The ASTM membership also works to harmonize its standards with those established by the International Standards Organization (ISO). By development and establishment of uniform standards of composition and performance of engineered tissues, the international community of care givers will have the greatest confidence and consistency in the introduction of advanced therapies for unserved medical needs. It would also be expected that these standards would promote the development of the maximum number of new therapies in the minimum amount of time, and at the lowest cost. Therefore, it may be predicted that the development by the international community of consensus standards for composition and performance of engineered skin will result in the greatest reductions in morbidity and mortality of patients with skin wounds.

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