Chimeric Human Skin Substitute Tissue: A Novel Treatment Option for the Delivery of Autologous Keratinocytes

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Background: For patients suffering from catastrophic burns, few treatment options are available. Chimeric coculture of patient-derived autologous cells with a “carrier” cell source of allogeneic keratinocytes has been proposed as a means to address the complex clinical problem of severe skin loss.

The Problem: Currently, autologous keratinocytes are harvested, cultured, and expanded to form graftable epidermal sheets. However, epidermal sheets are thin, are extremely fragile, and do not possess barrier function, which only develops as skin stratifies and matures. Grafting is typically delayed for up to 4 weeks to propagate a sufficient quantity of the patient’s cells for application to wound sites.

Basic/Clinical Science Advances: Fully stratified chimeric bioengineered skin substitutes could not only provide immediate wound coverage and restore barrier function, but would simultaneously deliver autologous keratinocytes to wounds. The ideal allogeneic cell source for this application would be an abundant supply of clinically evaluated, nontumorigenic, pathogen-free, human keratinocytes. To evaluate this potential cell-based therapy, mixed populations of a green fluorescent protein-labeled neonatal human keratinocyte cell line (NIKS) and unlabeled primary keratinocytes were used to model the allogeneic and autologous components of chimeric monolayer and organotypic cultures.

Clinical Care Relevance: Relatively few autologous keratinocytes may be required to produce fully stratified chimeric skin substitute tissue substantially composed of autologous keratinocyte-derived regions. The need for few autologous cells interspersed within an allogeneic “carrier” cell population may decrease cell expansion time, reducing the time to patient application.

Conclusion: This study provides proof of concept for utilizing NIKS keratinocytes as the allogeneic carrier for the generation of bioengineered chimeric skin substitute tissues capable of providing immediate wound coverage while simultaneously supplying autologous human cells for tissue regeneration.

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Abbreviations and Acronyms

GFP = green fluorescent protein

NIKS = neonatal human keratinocyte cell line

BACKGROUND

HUMAN SKIN PERFORMS MANY important functions, such as protecting against environmental insults, regulating body temperature, preventing invasion of infectious pathogens, and providing a barrier to transcutaneous water loss. Skin consists of two structural and functional layers: a self-renewing epidermis and a

supportive dermis separated by a basement membrane. The epidermis, the thinnest and outermost layer, is composed primarily of keratinocytes that terminally differentiate, forming an epithelial tissue with distinct morphological and biochemical characteristics related to its protective function. The underlying dermis is composed of fibroblasts embedded

within an extracellular matrix. The interfollicular epidermis is continuous with the cutaneous appendages (hair follicles, sebaceous, and sweat glands), which extend deep into the dermis. With severe skin injuries and extensive burns, disruption of the epidermal permeability barrier must be addressed promptly to prevent life-threatening complications. The standard of care for treatment of severe cutaneous trauma typically utilizes cadaver skin or synthetic dressings to achieve temporary coverage of the wound site. Cadaver skin or wound dressings function to prevent infection and dehydration, while providing the wound bed time to heal until the eventual acceptance of an autologous skin graft.¹ Permanent wound closure is generally accomplished through split-thickness autografting. Significantly, tissue harvest for autologous grafts is not feasible for patients suffering from extensive burns, as potential donor sites for autograft harvest are limited.

TARGET ARTICLE

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CLINICAL PROBLEM ADDRESSED

In the United States, approximately 45,000 people are hospitalized annually for burns² and approximately 10% of these patients require skin grafts.³ For patients suffering from catastrophic burns, one of the few available treatment options requires isolation of autologous keratinocytes from biopsies of unwounded tissue and subsequent culturing and expansion to form graftable epidermal sheets. However, widespread clinical success is hampered by technical challenges.⁴ Epidermal sheets, which are only a few cell layers thick, are extremely fragile and do not possess barrier function.⁵ Further, 3–4 weeks are required to culture sufficient quantities for application. In contrast to epidermal sheets, multilayered, fully stratified therapeutic human skin substitutes have been developed using organotypic tissue engineering strategies.^{6–8} The availability of bioengineered skin tissue with mechanical properties and barrier function approaching those of normal skin would be a significant improvement over current therapeutic options.

Chimeric culture of autologous and allogeneic cells has been proposed as an alternative therapy to address the complex clinical problem of se-

vere skin loss.^{9,10} As reviewed by Mansbridge,¹¹ the combined use of autologous and allogeneic cell sources could minimize the extent of culturing required, thus reducing the time to patient application. The use of relatively few autologous keratinocytes interspersed within an allogeneic “carrier” cell source is particularly attractive when considered for the formation of fully stratified skin substitutes. Reduced time for expansion of cells before organotypic culture further reduces the time to patient application of a fully stratified skin substitute that could prove superior to simplistic epidermal sheets. Minimizing both the time to treatment and the number of operative procedures required to achieve wound closure are substantial benefits to patients suffering from severe burns. The primary impediment to the generation of chimeric cultures for clinical use has been the lack of a reliable source of pathogen-free, allogeneic keratinocytes to serve as the carrier population.

RELEVANT BASIC SCIENCE CONTEXT

The feasibility of cocultured skin substitutes was demonstrated in a series of experiments using animal models. Chimeric cultures generated from both mouse and human keratinocytes resulted in the elimination of human cells from the wound site with retention of the mouse cells when grafted onto mice.¹⁰ Similarly, keratinocytes from different strains of mice (BALB/c and C3H/He) were cocultured and grafted onto either BALB/c or C3H/He mice. Syngeneic keratinocytes, donor cells isolated from the same strain as the recipient mouse, demonstrated long-term survival, whereas allogeneic keratinocytes, wherein donor and recipient strains differed, were rejected.⁹ Retention and long-term survival of syngeneic cells were demonstrated in chimeric cultures composed of up to 98% allogeneic and 2% syngeneic keratinocytes.¹² Translation of the chimeric approach for human therapeutic application was first reported in 2009 using NIKS epidermal progenitor cells, a neonatal human keratinocyte cell line, as the allogeneic cell source.¹³

EXPERIMENTAL MODEL OR MATERIAL: ADVANTAGES AND LIMITATIONS

As previously mentioned, the critical limitation of the chimeric autologous/allogeneic therapeutic approach for the treatment of severe skin loss has been the lack of an abundant source of allogeneic human keratinocytes suitable for therapeutic use. The genetically uniform, nontumorigenic, pathogen-free NIKS cell source is ideally suited for cell-based

therapies. These cells exhibit keratinocyte-specific growth and differentiation characteristics, yet display an extended lifespan *in vitro*.¹⁴ When grown in organotypic culture, these keratinocytes form a fully stratified epithelium possessing barrier function and tensile strength similar to that of intact skin. Allogeneic skin substitutes generated with NIKS keratinocytes have been evaluated in a Phase I/IIa clinical trial for the temporary management of traumatic skin wounds.^{15,16} NIKS-based skin substitutes were found to be comparable to the standard of care of cadaver skin, were well tolerated, and did not elicit acute allogeneic immune responses to NIKS cells in treated patients. No therapy-related adverse events were observed.

To assess the potential for NIKS keratinocytes to serve as a “carrier” for autologous keratinocytes, NIKS and primary keratinocytes isolated from neonatal skin were used to model the allogeneic and autologous components of chimeric monolayer and organotypic cultures (see Summary Illustration section). By exploiting the unique property of NIKS cells to be genetically engineered using nonviral methods, the carrier cell population was identified by labeling with green fluorescent protein (GFP). NIKS cells stably transfected to express GFP (NIKS^{GFP}) enabled clear distinction between fluorescent NIKS^{GFP} cells and nonfluorescent primary keratinocytes, allowing cell populations to be tracked in studies that examined the development and *in vivo* performance of fully stratified, biologically active chimeric skin substitutes.

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

NIKS^{GFP} and primary keratinocytes form contiguous epithelial sheets in monolayer culture

To determine whether NIKS^{GFP} cells impacted growth of primary keratinocytes, mixed populations of NIKS^{GFP} cells and unlabeled primary keratinocytes in proportions of 50%:50%, 10%:90%, or 1%:99% (NIKS^{GFP}:primary keratinocytes) were grown in monolayer tissue culture (see panel **A** in Summary Illustration figure). Cultures composed of 100% NIKS^{GFP} or 100% primary keratinocytes served as controls. Indirect immunofluorescent detection of the cell adhesion protein E-cadherin was used to assess the formation of cell–cell junctions between the two cell populations. Mixed cultures of NIKS^{GFP} cells and primary keratinocytes formed contiguous chimeric epithelial sheets exhibiting mature cell–cell junctions. Coculturing NIKS^{GFP} cells and primary keratinocytes did not

affect the growth rate of either strain ($p < 0.05$) in monolayer culture.

Two cell sources form chimeric skin tissue with barrier function comparable to intact skin

For subsequent studies, composite skin substitute tissues were generated using organotypic culturing methods. Mixed populations of NIKS^{GFP} cells and unlabeled primary keratinocytes were seeded onto contracted collagen type 1 matrices containing human dermal fibroblasts and maintained at the air/medium interface to facilitate formation of three-dimensional skin tissues (see panel **B** in Summary Illustration figure). Proportions of 90%:10% and 50%:50% (NIKS^{GFP}:primary keratinocytes) were evaluated, whereas tissues composed of 100% NIKS^{GFP} or 100% primary keratinocytes served as controls. Histological analysis confirmed the formation of tissue architecture typical of human epidermis for all chimeric and control skin substitutes. Tissue chimerism was confirmed by fluorescence microscopy. Within chimeric skin substitutes, discrete contiguous regions of fluorescing NIKS^{GFP} cells were clearly discernable from nonfluorescing tissue generated from primary keratinocytes. Fluorescing and nonfluorescing regions formed a patterned array of cell stacks that could be attributed to terminal differentiation of labeled or unlabeled proliferative keratinocytes. Of particular note, primary keratinocytes in chimeric tissues contributed substantially to the epidermis despite input percentages of 10% or 50%, suggesting that fluorescing cells were being displaced. The capacity of the chimeric system to form a functional epidermis was evaluated by examining the barrier properties of chimeric skin substitute tissues as measured by skin surface electrical impedance. Chimeric composite skin substitutes generated from coculture of NIKS^{GFP} and primary keratinocytes formed fully stratified epidermal tissue with barrier function similar to that of native human skin.

Appropriate proteins are expressed in the epidermal compartment of chimeric skin substitutes

Cell–cell adhesion proteins and proteins associated with keratinocyte terminal differentiation are essential to the structure and function of the epidermis. Indirect immunofluorescence analysis was used to evaluate expression and localization of these proteins within control and chimeric skin substitute tissues. No differences in either protein level or localization pattern were detected when chimeric tissues were compared with controls. Further analysis indicated that the extent of tissue

chimerism did not affect the expression of these critical proteins, as both GFP-labeled and non-labeled cells within the tissue were found to express the examined proteins. Therefore, proteins associated with cell–cell adhesion and keratinocyte terminal differentiation were found to be appropriately expressed and localized in chimeric composite skin substitutes.

Chimeric skin tissue stably engrafts and persists after surgical placement

Engraftment of chimeric skin substitute tissues was evaluated by surgically applying tissues to an excisional wound created on the dorsum of immunocompromised athymic nude mice. The 75–100 μm -thick, fully stratified skin substitute tissues were easily handled during surgical application. Wound sites were visually evaluated over the course of 3 months and biopsies were taken at 7, 14, 28, and 91 days after surgery. All skin substitute tissues engrafted and both chimeric and control tissues displayed a consistent appearance throughout the course of the study. Histological analysis, as evidenced by tissue architecture and cell morphology, revealed a seamless junction between engrafted human tissue and the surrounding mouse skin. The presence of human tissue was confirmed by direct immunofluorescent detection of the major histocompatibility complex class I human leukocyte antigen complex. Specific detection of human tissue indicated that chimeric skin substitutes stably engrafted *in vivo* and persisted for up to 3 months.

In the murine model, primary keratinocytes displace the NIKS^{GFP} cell population by 28 days after surgical placement

Fluorescence microscopy was employed to evaluate the pattern of GFP expression in engrafted skin substitute tissues generated from either 50%:50% or 90%:10% NIKS^{GFP}:primary keratinocytes or from 100% NIKS^{GFP} cells. In all tissues, GFP-labeled cells were detected in a dose-dependent manner at both 7 and 14 days after surgical placement. By 28 days, although GFP-labeled cells were detected in 100% NIKS^{GFP} tissues, no fluorescent cells were detected in chimeric tissues (see panel C in Summary Illustration figure). Absence of GFP-labeled cells suggested loss of the NIKS^{GFP} cell population through desquamation, with concomitant retention of unlabeled primary keratinocytes. To confirm that this observation resulted from absence of NIKS^{GFP} cells and not simply loss of the fluorescent protein, polymerase chain reaction was employed to detect the presence of the gene encoding GFP in genomic DNA isolated from chimeric tissues engrafted for 28 days. This anal-

ysis confirmed that engrafted human tissue did not contain the GFP marker; therefore, the primary keratinocyte population had displaced tissue composed of the NIKS^{GFP} carrier cell population. These results support the clinical use of fully stratified chimeric skin substitute tissue produced from NIKS keratinocytes interspersed with autologous, patient-derived keratinocytes for the treatment of severe skin injury such as catastrophic burns.

INNOVATION

Transplantation of fully stratified, biologically active chimeric skin substitute tissues represents an innovative regenerative medicine approach to the surgical management of severe skin trauma. Chimeric tissues not only provide immediate coverage to severe skin wounds and promote tissue regeneration, but simultaneously deliver autologous keratinocytes directly to the wound site. Availability of an abundant, well-characterized allogeneic cell source is critical for success. The NIKS progenitor cells, a genetically uniform, non-tumorigenic, pathogen-free source of keratinocytes that have been already clinically tested within the context of a skin substitute, are the ideal allogeneic cell source for this application.

SUMMARY ILLUSTRATION

In this study, mixed populations of NIKS^{GFP} and primary keratinocytes were cultivated together to

model the allogeneic and autologous components within chimeric cultures. As depicted in (A), monolayer culturing methods, wherein cells are propagated submerged under growth medium, were used to evaluate how the two cell populations interacted as they formed a contiguous epidermal sheet. For monolayer studies, cells were plated at ratios of 50%:50%, 10%:90%, or 1%:99% (NIKS^{GFP}:primary keratinocytes), with cultures of 100% NIKS^{GFP} or 100% primary keratinocytes as controls. Cultures were analyzed after defined time points or at confluence.

Organotypic culturing, wherein cells are maintained at the air/medium interface to mimic how human skin typically grows, was used to assess the interaction of the GFP-labeled and unlabeled populations as multilayered, fully stratified skin substitutes were formed (B). For chimeric studies, proportions of 90%:10% and 50%:50% (NIKS^{GFP}:primary keratinocytes) were seeded onto contracted collagen type 1 matrices containing human dermal fibroblasts. Control tissues were generated using either 100% NIKS^{GFP} or 100% primary keratinocytes. Tissues were initially kept submerged for 5 days to permit epithelialization of the dermal surface, after which they were raised to the air/medium interface and maintained for 18 days to enable differentiation and stratification.

Chimeric skin substitute tissues were surgically applied to an excisional wound created in an immunocompromised athymic nude mouse model. As depicted in (C), although GFP-labeled cells were initially detected after engraftment, no fluorescent cells were detected in engrafted chimeric tissues by 28 days after surgical application. This pattern suggested loss of the GFP-labeled cells through desquamation, whereas studies confirmed that unlabeled primary keratinocytes were retained.

CAUTION, CRITICAL REMARKS, AND RECOMMENDATIONS

Tissue engineering strategies have shown tremendous promise in the treatment of acute wounds.⁷ However, these cell-based therapies also bring new challenges, including complex and costly production processes and strict regulatory scru-

TAKE-HOME MESSAGE

Basic science advances

- Human keratinocyte progenitors can function as an allogeneic carrier for chimeric coculture.
- NIKS cells, stably transfected to express GFP using nonviral methods, enabled clear distinction between fluorescent NIKS^{GFP} cells and nonfluorescent primary keratinocytes.
- Monolayer coculture of two cell sources forms a contiguous chimeric epithelial sheet without negatively impacting on the growth rate.
- Chimeric skin substitutes possess normal tissue architecture, express proteins essential for the structure and function of epidermal tissue, and exhibit barrier function comparable to normal human skin.
- Chimeric skin substitutes stably engraft in an *in vivo* mouse model with long-term retention of primary keratinocytes and loss of NIKS^{GFP} keratinocytes.

Clinical science advances

- The genetically uniform, nontumorigenic, pathogen-free NIKS progenitor cell source is ideally suited for cell-based therapies.
- A Phase I/IIa safety and early efficacy clinical trial was conducted to assess full-thickness skin substitute tissues produced using unmodified NIKS keratinocytes in the temporary management of traumatic skin wounds. No acute immune responses to NIKS cells were found and no therapy-related adverse events occurred in treated patients.
- NIKS keratinocytes can function as an allogeneic carrier in the format of an engineered chimeric skin substitute tissue.

Relevance to clinical care

- Relatively few autologous keratinocytes may be required to produce fully stratified chimeric skin substitute tissue with substantial tissue regions attributable to the original patient-derived cells.
- The need for fewer autologous cells may decrease the clonal expansion time, reducing the time to patient application.
- Generation of a skin substitute that possesses cutaneous barrier function is a significant improvement over the application of autologous cell products lacking this important characteristic.

tiny. The level of complexity increases when the introduction of a specific patient's cells puts further burden on the production process. As each chimeric skin substitute is specific for a given patient, a degree of variability between the extents of carrier population displacement is anticipated. Each patient's age and health could impact on the proliferative capacity of the isolated keratinocytes, resulting in greater or fewer autologous cells within the final chimeric tissue. Although additional experimental studies to examine these properties must be performed using keratinocytes isolated from a broad range of potential patients, this challenge will remain as chimeric tissues transition to clinical use. Moreover, regulation of

this type of personalized cell-based therapeutic for use in wound healing is challenging. Aspects of both standard cell-based therapies and transplantation could apply. As personalized medicine progresses, these regulatory hurdles will need to be addressed.

FUTURE DEVELOPMENT OF INTEREST

The key factor to success for the chimeric skin tissue approach is the availability of a suitable, abundant, allogeneic cell source. Although it is anticipated that initial clinical evaluation of this therapeutic approach would utilize unmodified NIKS keratinocytes, as postulated in 1999 by Sheridan and coworkers, chimeric skin substitutes produced using autologous keratinocytes and genetically modified allogeneic cells could enhance tissue engraftment through provision of factors known to promote wound healing.¹⁷ The expression of wound healing factors from keratinocytes has traditionally required viral transduction,^{18,19} an approach that yields a heterogeneous population with a broad range of exogenous protein expression levels. As demonstrated by the successful generation and utilization of NIKS^{GFP} and the

work of Thomas-Virnig *et al.*,²⁰ NIKS progenitor cells are amenable to genetic engineering using nonviral means. It is anticipated that autologous cells could be interspersed within a background tissue designed to express and secrete any number of proteins targeting wound healing enhancement. These enhanced chimeric skin tissues represent the next generation of personalized therapeutic approaches to treat severe skin trauma and deliver autologous keratinocytes directly to the wound site.

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AUTHOR DISCLOSURE AND GHOSTWRITING

Cathy Rasmussen is an employee of Stratatech Corporation. Lynn Allen-Hoffmann is the founder and CEO of Stratatech Corporation.

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