Published in final edited form as:

J Trauma. 2009 March ; 66(3): 866–874. doi:10.1097/TA.0b013e31819849d6.

Phase I/II Clinical Evaluation of StrataGraft: A Consistent, Pathogen-Free Human Skin Substitute

Michael J. Schurr, MD¹, Kevin N. Foster, MD², John M. Centanni, MS³, Allen R. Comer, PhD³, April Wicks, BS³, Angela L. Gibson, PhD⁴, Christina L. Thomas-Virnig, PhD^{3,4}, Sandy J. Schlosser, BS⁴, Lee D. Faucher, MD¹, Mary A. Lokuta, PhD³, and B. Lynn Allen-Hoffmann, PhD^{3,4}

¹ Department of Surgery, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin

² Department of Surgery, Arizona Burn Center at Maricopa Medical Center, Phoenix, Arizona

³ Stratatech Corporation, Madison Wisconsin

⁴ Department of Pathology and Laboratory Medicine, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin

Abstract

Background—Large wounds often require temporary allograft placement to optimize the wound bed and prevent infection until permanent closure is feasible. We developed and clinically tested a second-generation living human skin substitute (StrataGraft). StrataGraft provides both a dermis and a fully-stratified, biologically-functional epidermis generated from a pathogen-free, long-lived human keratinocyte progenitor cell line, Neonatal Immortalized KeratinocyteS (NIKS).

Methods—Histology, electron microscopy, quantitative polymerase chain reaction, and bacterial growth in vitro were used to analyze human skin substitutes generated from primary human keratinocytes or NIKS cells. A phase I/II, National Institute of Health-funded, randomized, safety, and dose escalation trial was performed to assess autograft take in 15 patients 2 weeks after coverage with StrataGraft skin substitute or cryopreserved cadaver allograft.

Results—StrataGraft skin substitute exhibited a fully stratified epidermis with multilamellar lipid sheets and barrier function as well as robust human β defensin-3 mRNA levels. Analysis of the primary endpoint in the clinical study revealed no differences in autograft take between wound sites pretreated with StrataGraft skin substitute or cadaver allograft. No StrataGraft-related adverse events or serious adverse events were observed.

Conclusions—The major finding of this phase I/II clinical study is that performance of StrataGraft skin substitute was comparable to cadaver allograft for the temporary management of complex skin defects. StrataGraft skin substitute may also eliminate the risk for disease transmission associated with allograft tissue and offer additional protection to the wound bed through inherent antimicrobial properties. StrataGraft is a pathogen-free human skin substitute that is ideal for the management of severe skin wounds before autografting.

Address for reprints: Michael Schurr, MD, University of Wisconsin Hospital, 600 Highland Avenue, Madison, WI 53792; email: E-mail: schurr@surgery.wisc.edu.

Presented at the 67th Annual Meeting of the American Association for the Surgery of Trauma, September 24–27, 2008, Maui, Hawaii.

Ms. Lynn Allan-Hoffmann, PhD, is a founder and consultant to Stratatech Corporation. The company owns a proprietary interest in the StrataGraft® tissue production process and StrataLife™ culture media.

Keywords

Burn; Cadaver; Infection; Autograft; Allograft

Skin provides an essential role in fluid homeostasis and prevention of infection. Widespread full-thickness loss of skin integrity is common after trauma, burns, and necrotizing soft-tissue infections and patients suffer from the inherent loss of barrier function resulting in massive fluid losses and invasive bacterial infections. Current standards of care recommend resurfacing of the patient as quickly as possible with autologous skin grafts to restore fluid homeostasis and prevent sepsis. Unfortunately, this is often delayed due to patient instability, malnutrition, ongoing infection, or because of insufficient healthy donor skin available for wound site coverage.

Meshed cadaver skin is commonly used to promote vascularization and reduce bacterial bioburden in those cases where immediate autografting is not a viable option.^{1,2} Unfortunately, the cadaver skin supply is often contaminated, precluding its use and presenting serious safety concerns because of the risk of disease transmission.^{3,4} Physicians often must rely on cryopreserved cadaver skin which has limited viability. Development of new treatment alternatives are needed to provide safer, consistent, and more available wound dressings for complex skin wounds. The potential advantages to patient health are to eliminate the risk of disease transmission and to provide a readily available, more consistent allograft with biologically active keratinocytes for clinical use.

The first generation of skin substitutes containing living cells were promising treatment options for the temporary coverage of cutaneous wounds. Replacement of both dermal and epidermal compartments is paramount and living skin substitutes are accepted as more beneficial than acellular or biosynthetic skin substitutes.⁵ However, cell sourcing for these skin substitutes is a well-recognized problem directly coupled to safety concerns.⁶ In addition, many of the first-generation living skin substitutes have poor handling characteristics and typically lack epidermal barrier function.^{7,8} The rapid deterioration observed for some of them in the wound bed⁹ may compromise the delivery of growth factors and other desirable bioactive molecules.

For patients with large wounds, a living skin substitute with a multilayered, mature epidermis may restore the skin's barrier function and deliver bioactive molecules for wound bed conditioning and tissue regeneration. Recent findings suggest that host defense peptides (HDPs) such as human β -defensins (hBD) are central to keratinocyte-mediated killing of pathogens. hBDs have potent antimicrobial activity and constitutive hBD-3 expression in the suprabasal layers is enhanced during epidermal differentiation of keratinocytes.¹⁰ Exposure to *Staphylococcus aureus* (*S. aureus*) induces hBD-3 cutaneous expression and this induction is associated with increased antimicrobial activity.^{11,12} A link between cutaneous hBD-3 levels and bacterial growth is also seen in atopic dermatitis where hBD-3 deficiency appears to be causally associated with *S. aureus* skin infections.¹³ Human skin substitutes possessing a mature, viable, fully-stratified epidermis may forestall infection through the expression and processing of HDPs.

The characteristics of ideal wound dressings or biological skin substitutes were first suggested by Pruitt and Levine¹⁴ and have been expanded upon by others in recent years (Table 1)^{7,9}. StrataGraft is a second-generation living skin substitute which possesses many of these characteristics. It contains both epidermis and dermis with tensile strength and barrier function comparable to that of intact human skin. The epidermal layer of StrataGraft skin substitute is generated from NIKS cells, a consistent source of pathogen-free human keratinocyte progenitor cells which are amenable to stable genetic modification with nonviral vectors (Thomas-Virnig

et al.,¹⁵ and Rasmussen et al. [unpublished]). In this report, StrataGraft skin substitute was compared with cadaver allograft in patients undergoing sequential skin reconstruction procedures before autograft placement. The trial was conducted under an Investigational New Drug application with the US Food and Drug Administration (FDA).

PATIENTS AND METHODS

Cell Isolation, Culture, and Tissue Production

Normal human epidermal keratinocytes (NHEK) strains BC-1-Ep and GS-1-Ep were isolated from newborn human foreskin tissue and cultured as described.¹⁶ Samples were obtained with informed consent and studies were approved by both Meriter Hospital (Madison, WI) and the University of Wisconsin Human Subjects Committee Institutional Review Boards. The NIKS cell line was isolated from a senescing culture of BC-1-Ep keratinocytes.¹⁷ Studies comparing NIKS and NHEK tissue were conducted at the University of Wisconsin School of Medicine and Public Health.

StrataGraft tissue for clinical use was produced at the Waisman Clinical Biomanufacturing Facility in accordance with current Good Manufacturing Practices. The StrataGraft tissue production process and StrataLife culture media are proprietary to Stratatech Corporation and were optimized to promote epidermal stratification and barrier function. All cells, media, and reagents were tested for potential adventitious agents before release for use in tissue production. Dermal equivalents were prepared by combining normal human dermal fibroblasts with a nonbovine source of purified type I collagen in Transwell tissue culture inserts (Corning, Corning, NY). NIKS cells were thawed from a cryopreserved cell bank and expanded before seeding onto dermal equivalents and later lifted to expose the tissue surface to the humidified culture environment. Before clinical use, each StrataGraft tissue lot was tested with a proprietary panel of tests to ensure the sterility, identity, purity, and potency of StrataGraft.

Histology Preparation

StrataGraft tissue was fixed for 2 hours in 1% paraformaldehyde in phosphate-buffered saline or 10% buffered formalin and processed for paraffin embedding using a Sakura Tissue-Tek VIP processor. Paraffin sections (5 μ m) were stained with hematoxylin and eosin to evaluate tissue morphology and imaged using an Olympus IX-71 microscope and DPController software. Sections of StrataGraft skin substitute were processed for electron microscopy (EM) as described with minor modifications.¹⁸ Briefly, samples were fixed in Karnovsky's fixative and 50 μ m vibratome sections were cut. Samples postfixed sequentially with 1% osmium tetroxide, washed with phosphate-buffered saline, and postfixed with 0.5% ruthenium tetroxide (Polysciences, Warrington, PA). Samples dehydrated in 70% EtOH and infiltrated with 70% hydroxypropyl methacrylate (Electron Microscopy Sciences, Fort Washington, PA).¹⁹ Freshly prepared Eponate 12 (Ted Pella, Redding, CA) was used for embedding and polymerization. Thin sections were cut on a Reichert Ultracut E (Leica Microsystems, Buffalo, NY). EMs were captured on Eastman Kodak 4489 film (Rochester, NY) using a Hitachi H-7000 electron microscope (San Jose, CA) operated at 75 kV.

RNA Isolation and Quantitative Polymerase Chain Reaction Analysis

Total RNA was isolated from NIKS and NHEK tissues using Trizol (Gibco/Invitrogen, Carlsbad, CA). After DNase I treatment of 1 μ g total RNA (Ambion, Austin, TX), reverse transcription was performed using oligo-dT primers and Maloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen, Carlsbad, CA). TaqMan Gene Expression Assays probe and primer pairs for hBD-3 (Assay ID: Hs00218678_m1) and human peptidylprolyl isomerase A Endogenous Control (cyclophilin A; assay ID: Hs99999904_m1) were from Applied Biosystems (Foster City, CA). Quantitative polymerase chain reaction analysis was performed

in three independent experiments each run in triplicate on the Bio-Rad Chromo4 Real-time Polymerase Chain Reaction Detection System (Hercules, CA). hBD-3 expression was normalized to cyclophilin²⁰ mRNA expression levels. Opticon Monitor Software and the Genex Excel Macro (Bio-Rad) were used for data analysis.

In Vitro Antimicrobial Assay

Organotypic tissue was submerged in StrataLife medium for 24 hours. The tissue was then removed from the insert and StrataLife medium containing 10^4 /mL *Staphylococcus carnosus* (grown to log phase and diluted in Trypticase Soy Broth mix) was added in a 1:1 ratio to the media and tissue already present in the well and incubated for 2 hours at 37°C. Samples were diluted and spread on Trypticase Soy Agar plates using a WASP2 spiral plater (Microbiology International, Frederick, MD), and incubated at 37°C for 18 hours. The colony forming units (CFU)/mL was calculated.

Clinical Trial Methods

Study Enrollment, Inclusion, and Exclusion Criteria—A phase I/II, multicenter, open-label, randomized, safety, and dose escalation trial (NCT00618839) was conducted under a clinical protocol approved by the Center for Biologics Evaluation and Research (CBER) at the FDA and monitored by a Data and Safety Monitoring Board. This trial was conducted in accordance with Good Clinical Practices (GCP) and FDA regulations and guidelines. These regulations and guidelines encompass all principles established by the Declaration of Helsinki and all its subsequent amendments. The protocol and informed consent forms were approved by the respective IRB for each participating study site. The consent form was available in English and Spanish as needed. At the time of enrollment, all patients were assigned a sequential, de-identified number and clinical study site identifier. For the purposes of maintaining patient anonymity and to eliminate bias, all patient samples and representative photos were labeled with the coded patient number and the blinded wound site designation.) The study was performed to assess autograft take and infection in 15 patients with full-thickness skin defects, who were intended to undergo sequential surgical procedures involving surgical skin debridement and temporary cadaver allograft placement followed by autografting. The trial was conducted at the University of Wisconsin Hospital and Clinics (Madison, WI) and the Arizona Burn Center at Maricopa Medical Center (Phoenix, AZ). Informed consent was obtained for all study subjects before enrollment into the trial. The study population included male and female patients, 18 years or older, with full-thickness skin defects $\geq 5\%$ total body surface area (TBSA). Pregnant or lactating women, or patients with active malignancy, undergoing immunosuppressive therapy, evidence of malnutrition, clinically significant organ system injury (renal, hepatic, cardiac, pulmonary, or neurologic), or life expectancies < 3 months, were excluded from the study.

Patient Treatment and Study Design

Treatment for each patient was randomized such that each half of the wound site received StrataGraft or cadaver allograft. Cryopreserved cadaver skin was obtained from an AATB-certified tissue bank. The allogeneic skin treatments were applied to the debrided wound site for 1 week to condition the wound bed. After placement of the allografts, each half wound site was assessed for appearance and visual signs of infection every other day until allograft removal 1 week after placement. Autograft placement occurred when the wound bed was suitable to accept an autograft. Percent autograft take was assessed 2 weeks after autograft placement. Autograft healing and wound site infection was assessed at 1, 2, 3, 4, 8, and 12 weeks post-placement. As additional patients were enrolled, the targeted dose of StrataGraft allograft was increased. The first five patients were treated with up to 0.5% TBSA of StrataGraft tissue. The next five patients were treated with up to 1.0% TBSA of StrataGraft. The third group was

treated with up to 1.5% TBSA of StrataGraft. Adverse events (AE) and serious adverse events (SAE) were defined on a modified World Health Organization Toxicity Grading Scale for Determining the Severity of Adverse Events.

Clinical and Laboratory Tests

Clinical and laboratory tests performed included baseline organ system toxicities, laboratory studies, physical examinations, and vital signs. Laboratory studies included hematology, blood chemistry, urinalysis, and panel reactive antibody assessment before, during, and after treatment. AE and concomitant medications were recorded. Consistent with FDA policy, archival samples were collected including: serum, viable leukocytes, and plasma. At the time of allograft removal, archival biopsy samples were obtained. Photodocumentation of all visual assessments was obtained and archived.

Study Endpoints

The primary safety and efficacy outcome was the percentage of autograft take 2 weeks after autograft placement. Safety and secondary efficacy assessments were made based on allograft appearance, allograft adherence, evidence of infection, evidence of revascularization, and appearance of the wound site after autografting. Bacterial cultures were obtained from the wound bed after the initial wound debridement and at the time of allograft removal.

Statistical Analyses

Results are shown as mean $\pm$ SD. Differences between StrataGraft and cadaveric grafts were assessed with a paired, two-sided *t* test; *p* < 0.05 was considered significant and 95% confidence intervals for the differences in means are shown. Data plots were used to assess gross deviations from the assumptions of the paired *t* test. To avoid overplotting, some values were jittered (R for Windows v. 2.5.1).^{21,22} Repeated measures data for each patient were averaged over sessions. Comparisons of more than two groups were assessed by one-way analysis of variance, with Dunnett's post-hoc test for multiple comparisons of experimental groups to a single reference (Prism v.4). *p* values < 0.05 were considered statistically significant.

RESULTS

Skin Tissue Prepared From NIKS Keratinocytes Expresses Elevated Levels of hBD-3

hBD are one of the main groups of HDPs which are expressed in skin. Microarray results¹⁶ coupled with a recent report of copy number polymorphisms leading to inter-individual variability of hBD-3 expression²³ led us to examine the hBD-3 mRNA levels in tissue generated with NIKS compared with NHEK. Quantitative polymerase chain reaction analysis revealed increased hBD-3 mRNA levels (approx. 8-fold) in NIKS cells compared with BC-1-Ep keratinocytes, the parental strain from which the NIKS cells were derived, and an independent strain of NHEKs (GS-1-Ep) (Fig. 1). The higher level of hBD-3 mRNA led us to investigate the effect this differential expression may have on the ability of NIKS tissue to inhibit bacterial growth in an antimicrobial activity assay.

Skin Tissue Prepared From NIKS Keratinocytes Exhibits Enhanced Antimicrobial Activity

Antimicrobial activity mediated by hBD-2 and hBD-3 has been demonstrated in cell lysates of human keratinocytes exposed to *S. aureus*.¹² We determined whether the higher levels of hBD-3 mRNA observed in NIKS skin substitute tissue was associated with enhanced antimicrobial activity. *S. carnosus*, a staphylococcal species that is nonadherent and not internalized upon association with keratinocytes,²⁴ was used in this assay since the pathogenic strains tested adhere firmly to the tissues resulting in artificially low levels of bacterial outgrowth. NIKS tissue reduced the number of CFU by more than 50% as compared with

tissues generated with NHEK strains (Fig. 2). These results indicate that skin substitutes prepared from NIKS keratinocytes inhibit growth of *S. carnosus*, more effectively than substitutes made with NHEK sources.

StrataGraft Clinical Investigation

We hypothesized that StrataGraft skin substitute would be comparable to cadaver skin in the treatment of debrided wounds before autograft placement. To test this hypothesis, we conducted a phase I/II, randomized, safety, and dose escalation trial to assess autograft take and infection in 15 patients whose wounds were temporarily grafted with cadaver and StrataGraft allograft before autografting. For these studies, StrataGraft skin substitute was manufactured according to current Good Manufacturing Practices procedures. StrataGraft is a tough, suturable, allogeneic skin substitute that is able to be meshed before placement (Fig. 3; 1:1 ratio, Brennan Medical, Saint Paul, MN). Cadaver allografts were meshed 2:1 before placement. As shown in Figure 4, A, StrataGraft consists of a fully stratified, epidermal layer attached to a dermal equivalent containing fibroblasts. The epidermal layer exhibits tissue-specific stratification and possesses basal, spinous, granular, and cornified layers. The formation of a competent epidermal permeability barrier within the stratum corneum is dependent upon the assembly of specialized lipid lamellae that fill the extracellular space between the corneocytes. After staining with ruthenium tetroxide, these lipid lamellae exhibit an alternating electron dense and lucent staining patterning as shown in the EM of the stratum corneum of StrataGraft skin substitute (Fig. 4, B).

Clinical Trial Demographics

Fifteen patients with full-thickness skin loss of $\geq 5\%$ TBSA who met the inclusion and exclusion criteria were enrolled into the phase I/II clinical trial from 2007 to 2008. The patients enrolled in this study are summarized in Table 2 and had wounds resulting from thermal burns ($n = 11$), surgical resection of soft tissue infection ($n = 2$), electrical burns ($n = 1$), and traumatic injury ($n = 1$). Mean patient age was $43.4 \text{ years} \pm 14.7 \text{ years}$; 73.3% of the patients were male, and the mean full-thickness skin loss was $22.2\% \text{ TBSA} \pm 18.2$.

Clinical Trial Safety and Efficacy

All patients completed the treatment and follow-up phases of the study. There were no deaths in this study and no allograft-related AE or SAE indicative of local or systemic toxicity from exposure to StrataGraft or cadaver skin. The primary safety and efficacy outcome was percentage autograft take, assessed 2 weeks after autograft placement. Analyses of the primary outcome data found that autograft take was equivalent in areas treated with StrataGraft skin substitute ($97.7\% \pm 6.78$) or cadaver skin ($96.7\% \pm 10.47$). The appearance of the wound sites after autografting was assessed using the following scale: healed, no further surgical intervention (2 points); unhealed, but better than on presentation (1 point); no improvement (0 points); or worse than on presentation (-1 point). Autograft appearance (mean of all assessments) was not significantly different between StrataGraft (1.80 ± 0.40) and cadaver (1.78 ± 0.42) allograft.

Additional secondary outcomes were assessed before autografting. Evaluations of StrataGraft and cadaver allograft appearance were performed every other day after placement and at the time of allograft removal. Both StrataGraft and cadaver allograft maintained physical integrity after placement. The following three point scale was used to assess the condition of skin allografts: pink and adherent (2 points); either pink or adherent but not both (1 point); or neither pink nor adherent (0 points). The appearance of StrataGraft human skin substitute was significantly better (1.70 ± 0.62) than that of cadaver skin (1.49 ± 0.65 ; $p < 0.010$; 95% CI for the cadaver-StrataGraft mean = -0.372 to -0.061). After 1 week in the wound bed, StrataGraft was consistently more pink and adherent and was deemed overall to be healthier than the

cadaver allograft (Fig. 5). Examination of StrataGraft skin substitute after removal from the wound bed shows it to be a consistent source of viable keratinocytes (data not shown).

After removal of the temporary allografts, the wound bed that had been covered by either StrataGraft human skin substitute or cadaver skin was evaluated by the following wound site appearance criteria: normal healthy wound bed suitable for autografting (1 point) or not suitable for autografting (2 points). Wound bed appearance before autografting was identical for both the StrataGraft (1.27 ± 0.46) and cadaver (1.27 ± 0.46) allograft-treated half wound sites. In 13 of 15 patients (87%), autograft was placed 1 week after study entry. However, in two patients, the wound was judged to be not suitable for autograft placement and definitive coverage was delayed by approximately 2 weeks. A beneficial characteristic of StrataGraft skin substitute is that it retained translucent properties which allowed for direct observation of the wound bed condition before removal (Fig. 5).

The wound sites were evaluated for infection at several points before removal of the allografts, and at several points after autograft placement. A three point scale was used to assess wound infection: no evidence of infection by visual or microbiological assessment (0 points); visual evidence of infection (inflammation or purulent exudates) but not confirmed by microbiological assessment (-1 point); or evidence of infection by microbiological assessment confirmed by clinical appearance (-2 points). There were no differences in infection assessment scores or in quantitative microbiological assessment between groups at baseline or at time of allograft removal (StrataGraft[®] and cadaver allograft values; -0.14 ± 0.47). All quantitative cultures analyzed contained $<10^5$ CFU/gram tissue.

DISCUSSION

The findings of this phase I/II clinical trial support the hypothesis that StrataGraft skin substitute is comparable to cadaver allograft in the temporary treatment of debrided wounds before autograft placement. This living skin substitute was equivalent to cadaver skin in terms of wound bed preparation and did not increase the incidence of wound infection. StrataGraft was easily meshed, became pink and adherent after placement, and remained intact in the wound bed. This second-generation allogeneic, living skin substitute generated from a consistent source of pathogen-free keratinocytes has the obvious advantages of availability, physical properties, and production of bioactive molecules that warrant further clinical development.

The major finding of this clinical study is that the success rate of autografts after treatment of the wound bed with StrataGraft skin substitute was equivalent to that of cadaver allograft, the current standard of care. However, in today's clinical practice, the success rate of skin autograft take using the current standard of care is high. Autograft loss is not a common clinical problem because clinicians ensure that the wound bed is suitable for autografting before subjecting the patient to a painful donor site wound. Autograft take was $>96\%$ in this trial, therefore we would be unable to demonstrate enhanced efficacy using autograft take as the primary efficacy assessment. However, it is possible that alternative outcome measures or additional time points may elucidate signs of improved or accelerated healing. For example, StrataGraft does not disintegrate in the wound bed like some first generation skin substitutes,⁹ therefore it is tempting to speculate that it may better condition the wound bed and promote wound healing by more consistent, sustained delivery of bioactive molecules to the wound.

Most patients with skin injury do not have large areas of full-thickness skin loss. The vast majority of all burns have less than 20% TBSA involvement.²⁵ Many burns and other diseases require intensive therapy and cause significant pain and morbidity despite that fact that the skin loss is only partial thickness and the wounds will heal without operative intervention. There

has been considerable interest during the years in the treatment of partial-thickness skin loss with cadaver allograft or living skin substitutes. Early studies with cultured allogeneic keratinocyte grafts were reported to speed healing of partial-thickness burns with an improved cosmetic outcome.^{26,27} The challenges associated with tissue sourcing, establishment and expansion of primary keratinocyte cultures coupled with the poor quality of the resulting cultured skin substitute has prompted other investigators to use cryopre-served cadaver allograft for the treatment of second degree burns post-debridement despite the inherent issues associated with cadaver allograft availability and quality. Shorter healing times and improved cosmetic outcomes have been well documented.^{28,29} Some have gone so far as to suggest that dermal scald burns should be surgically debrided and covered with allogeneic cultured keratinocytes as the treatment of choice for partial-thickness injury.³⁰ Given this, additional clinical study of the pathogen-free StrataGraft living skin substitute for use in patients with partial-thickness skin loss is a high priority.

The first generation of human skin substitutes have inherent challenges associated with the lack of genetic consistency in the primary keratinocyte strains sourced from different humans. Both known and unknown polymorphisms in bioactive molecules can affect not only key cell growth characteristics required for consistent manufacture of complex human skin substitutes but also the ultimate potency of the final product. Here, we show that StrataGraft skin tissue may offer additional protection to the wound bed through sustained production of endogenous bioactive molecules such as HDPs. The biological significance of NIKS skin substitute-mediated attenuation of bacterial growth remains to be established using clinically relevant pathogens in an in vivo model of infected skin wounds.

It has been suggested that the second generation of human skin substitutes should directly target the underlying pathophysiology of the wound.⁷ Proof of principal for this concept has been established in human skin substitutes using virally infected keratinocytes overexpressing vascular endothelial growth factor as a strategy to improve early vascularization.³¹ Similarly, virally-induced overexpression of platelet-derived growth factor has also been proposed as a means to achieve earlier cellular repopulation of the dermis.³² However, these strategies to enhance product potency required infection with viral vectors and were limited by the eventual senescence of the infected cells and concerns about safety.

The NIKS keratinocytes are a well-characterized, genetically identical source of human keratinocytes that are amenable to genetic modification with nonviral vectors. Recently, we have demonstrated that NIKS cells stably transfected with a nonviral vector encoding green fluorescent protein retain the ability to undergo normal epidermal differentiation in organotypic culture (Rasmussen et al., unpublished data). In addition, we have stably introduced a number of therapeutically relevant genes into the NIKS keratinocytes (Thomas-Virnig et al.¹⁵) The genetically modified NIKS cells have been passaged through multiple generations and have the same long-lived characteristics as the original NIKS cells. These results demonstrate that NIKS cells are amenable to a variety of genetic modifications that could have important clinical applications not only in acute partial and full-thickness skin loss situations, but also for patients with chronic wounds from diseases such as venous insufficiency or diabetes.

Based on the findings reported here, the pathogen-free human skin substitute StrataGraft is comparable to the standard of care for the temporary management of severe skin wounds before autografting. StrataGraft skin substitute exhibits many of the physical and biological properties identified as features of an ideal skin substitute.¹⁴ Clinical assessment of the StrataGraft-treated wound beds suggests that it may continue to function beyond time frame investigated. A study investigating longer exposure to StrataGraft is warranted given the safety and efficacy results we observed. In addition to use in full-thickness wounds examined in the current study,

StrataGraft skin substitute's unique characteristics and potential for genetic enhancement hold promise for the treatment of partial-thickness skin loss or chronic wounds.

Acknowledgements

We wish to acknowledge the dedicated nursing and research study staff including Cindy Schmitz, Deborah Gawin, Janice Yahey at the University of Wisconsin-Hospital and Clinics, and Karen Richey at the Arizona Burn Center at the Maricopa Medical Center. We would also like to thank Alejandro Munoz and Victoria Rajamanickam for their assistance with the statistical analyses and Carol Sattler for her electron microscopy expertise.

Supported by the following grants: Howard Hughes Medical Institute (to M.J.S. and L.A.H.), Stratatech Corporation, University of Wisconsin Department of Surgery (to M.J.S.), NIH/NIAMS R43-AR47499 (to A.R.C.), NIH/NHLBI R01-HL74284 (to B.L.A.-H.), NIH/NIAMS R41-AR050349 (to B.L.A.-H.), NIH/NIAMS R42-AR050349 (to B.L.A.-H.), NIH/NIAMS R44-AR47499 translational clinical grant (A.R.C.).

References

1. Batchelor JR, Hackett M. HL-A matching in treatment of burned patients with skin allografts. *Lancet* 1970;2:581–583. [PubMed: 4195544]
2. Greenleaf G, Cooper ML, Hansbrough JF. Microbial contamination in allografted wound beds in patients with burns. *J Burn Care Rehabil* 1991;12:442–445. [PubMed: 1752879]
3. Mathur M, De A, Gore M. Microbiological assessment of cadaver skin grafts received in a skin bank. *Burns* 2008;35:104–106. [PubMed: 18603376]
4. Barnett JR, McCauley RL, Schutzler S, Sheridan K, Hegggers JP. Cadaver donor discards secondary to serology. *J Burn Care Rehabil* 2001;22:124–127. [PubMed: 11302599]
5. MacNeil S. Progress and opportunities for tissue-engineered skin. *Nature* 2007;445:874–880. [PubMed: 17314974]
6. Griffith LG, Naughton G. Tissue engineering—current challenges and expanding opportunities. *Science* 2002;295:1009–1014. [PubMed: 11834815]
7. Hrabchak C, Flynn L, Woodhouse KA. Biological skin substitutes for wound cover and closure. *Expert Rev Med Devices* 2006;3:373–385. [PubMed: 16681458]
8. Eisenbud D, Huang N, Luke S, Silberklang M. Skin substitutes and wound healing: current status and challenges. *Wounds* 2004;16:2–17.
9. Ehrenreich M, Ruszczak Z. Update on tissue-engineered biological dressings. *Tissue Eng* 2006;12:2407–2424. [PubMed: 16995775]
10. Supp DM, Karpinski AC, Boyce ST. Expression of human beta-defensins HBD-1, HBD-2, and HBD-3 in cultured keratinocytes and skin substitutes. *Burns* 2004;30:643–648. [PubMed: 15475135]
11. Harder J, Bartels J, Christophers E, Schroder JM. Isolation and characterization of human beta-defensin-3, a novel human inducible peptide antibiotic. *J Biol Chem* 2001;276:5707–5713. [PubMed: 11085990]
12. Kisich KO, Howell MD, Boguniewicz M, Heizer HR, Watson NU, Leung DY. The Constitutive capacity of human keratinocytes to kill *Staphylococcus aureus* is dependent on beta-defensin 3. *J Invest Dermatol* 2007;127:2368–2380. [PubMed: 17460726]
13. Kisich KO, Carspecken CW, Fieve S, Boguniewicz M, Leung DY. Defective killing of *Staphylococcus aureus* in atopic dermatitis is associated with reduced mobilization of human beta-defensin-3. *J Allergy Clin Immunol* 2008;122:62–68. [PubMed: 18538383]
14. Pruitt BA Jr, Levine NS. Characteristics and uses of biologic dressings and skin substitutes. *Arch Surg* 1984;119:312–322. [PubMed: 6365034]
15. Thomas-Virnig CL, Centanni JM, Johnston CE, et al. Inhibition of multidrug-resistant *Acinetobacter baumannii* by non-viral expression of hCAP-18 in a bioengineered human skin tissue. *Mol Ther*. 2009In press
16. Gibson, AL. *Cell Mol Biol*. Madison: University of Wisconsin; 2007. Cutaneous wound therapy: a role for therapeutic skin substitutes; p. 239

17. Allen-Hoffmann BL, Schlosser SJ, Ivarie CA, Sattler CA, Meisner LF, O'Connor SL. Normal growth and differentiation in a spontaneously immortalized near-diploid human keratinocyte cell line, NIKS. *J Invest Dermatol* 2000;114:444–455. [PubMed: 10692102]
18. van der Meulen J, van den Bergh BA, Mulder AA, Mommaas AM, Bouwstra JA, Koerten HK. The use of vibratome sections for the ruthenium tetroxide protocol: a key for optimal visualization of epidermal lipid bilayers of the entire human stratum corneum in transmission electron microscopy. *J Microsc* 1996;184:67–70. [PubMed: 8923758]
19. Brinkley BR, Murphy P, Richardson LC. Procedure for embedding in situ selected cells cultured in vitro. *J Cell Biol* 1967;35:279–283. [PubMed: 4863049]
20. Steele BK, Meyers C, Ozburn MA. Variable expression of some “housekeeping” genes during human keratinocyte differentiation. *Anal Biochem* 2002;307:341–347. [PubMed: 12202253]
21. Gentleman, R.; Ihaka, RR. Version 2.5.1: Free Software Foundation, R Development Core Team; 2007. [Accessed September 18, 2008]. Available at: <http://www.R-project.org>
22. Wong, Y. Statistical Analysis: an Introduction using R. Wikibooks. 2007. Available at: http://en.wikibooks.org/wiki/Statistical_Analysis:_an_Introduction_using_R/Statistics_and_R
23. Hollox EJ, Armour JA, Barber JC. Extensive normal copy number variation of a beta-defensin antimicrobial-gene cluster. *Am J Hum Genet* 2003;73:591–600. [PubMed: 12916016]
24. Agerer F, Lux S, Michel A, Rohde M, Ohlsen K, Hauck CR. Cellular invasion by *Staphylococcus aureus* reveals a functional link between focal adhesion kinase and cortactin in integrin-mediated internalisation. *J Cell Sci* 2005;118:2189–2200. [PubMed: 15855238]
25. Latenser BA, Miller SF, Bessey PQ, et al. National burn repository 2006: a ten-year review. *J Burn Care Res* 2007;28:635–658. [PubMed: 17969244]
26. Hefton JM, Madden MR, Finkelstein JL, Shires GT. Grafting of burn patients with allografts of cultured epidermal cells. *Lancet* 1983;2:428–430. [PubMed: 6135914]
27. Madden MR, Finkelstein JL, Staiano-Coico L, et al. Grafting of cultured allogeneic epidermis on second- and third-degree burn wounds on 26 patients. *J Trauma* 1986;26:955–962. [PubMed: 3537324]
28. Eldad A, Din AE, Weinberg A, et al. Cryopreserved cadaveric allografts for treatment of unexcised partial thickness flame burns: clinical experience with 12 patients. *Burns* 1997;23:608–614. [PubMed: 9568334]
29. Horch RE, Jeschke MG, Spilker G, Herndon DN, Kopp J. Treatment of second degree facial burns with allografts—preliminary results. *Burns* 2005;31:597–602. [PubMed: 15993304]
30. Rab M, Koller R, Ruzicka M, et al. Should dermal scald burns in children be covered with autologous skin grafts or with allogeneic cultivated keratinocytes?—“The Viennese concept”. *Burns* 2005;31:578–586. [PubMed: 15993303]
31. Supp DM, Boyce ST. Overexpression of vascular endothelial growth factor accelerates early vascularization and improves healing of genetically modified cultured skin substitutes. *J Burn Care Rehabil* 2002;23:10–20. [PubMed: 11803307]
32. Eming SA, Medalie DA, Tompkins RG, Yarmush ML, Morgan JR. Genetically modified human keratinocytes overexpressing PDGF-A enhance the performance of a composite skin graft. *Hum Gene Ther* 1998;9:529–539. [PubMed: 9525314]

Fig. 1.

Human β -defensin-3 (hBD-3) expression is elevated at the mRNA level in NIKS skin substitute. RNA was isolated from skin substitutes generated using GS-1-Ep, BC-1-Ep, and NIKS keratinocytes and relative hBD-3 mRNA levels were determined by qPCR. Equal amounts of input RNA were used and cyclophilin RNA levels served as internal reaction controls. hBD-3 expression was normalized to endogenous cyclophilin mRNA levels and then to that of NHEK cultures. Data represent three independent experiments. Statistical significance was determined using one-way ANOVA with Dunnett's post-hoc test for multiple comparisons against a single reference (NIKS).

Fig. 2. NIKS tissue inhibits bacterial growth. Shown are the relative colony forming units (CFU)/mL of *Staphylococcus carnosus* incubated with submerged skin substitute tissues made from GS-1-Ep, BC-1-Ep, and NIKS keratinocytes. The average number of viable bacteria from three independent experiments is presented as the percentage of CFU/mL relative to the GS-1-Ep tissue. Statistical significance was determined using one-way ANOVA with Dunnett's post-hoc test for multiple comparisons against a single reference (NIKS).

Fig. 3. StrataGraft skin tissue. *Panel A* shows an intact StrataGraft skin substitute which has a surface area of 44 cm². Meshed StrataGraft skin substitute (1:1 ratio) is shown in *Panel B*.

Fig. 4.

Tissue morphology and lipid lamellae in StrataGraft skin substitute. (A) Tissue sections were fixed, embedded, and stained with hematoxylin and eosin. StrataGraft substitute consists of an epidermal layer attached to a dermal equivalent containing fibro-blasts. Within the epidermis, a single layer of highly ordered basal keratinocytes lies below several layers of spinous cells. Between the compact stratum corneum and the spinous cells is a layer of distinct granular cells, with prominent intracellular keratohyalin granules. Magnification = $\times 200$, scale bar = 200 μm . (B) Stratum corneum lipid lamellae from StrataGraft skin substitute were visualized by electron microscopy using ruthenium tetroxide. Lamellar structures with an alternating

electron-dense and lucent banding pattern were observed. Magnification = $\times 420,000$, scale bar = 100 nm.

Fig. 5.

Visual appearance of StrataGraft skin substitute in the wound bed. One week after placement in the wound bed, the StrataGraft skin substitute (upper portion of the wound bed) appears viable and adherent to the wound in comparison to the cadaver allograft (lower portion of the wound bed) in the same wound bed. Note that the translucent nature of StrataGraft skin substitute allows for visualization of the wound bed, in contrast to the opaque appearance of the cadaver allograft.

Table 1
Characteristics of the Ideal Biologic Skin Substitute*

Absence of antigenicity
Tissue compatibility
Absence of local or systemic toxicity
Impermeable to exogenous microorganisms
Water vapor transmission similar to normal skin
Rapid and sustained adherence to wound surface
Conformal to surface irregularities
Elastic to permit motion of underlying tissue
Resistant to linear and shear stresses
Tensile strength to resist fragmentation
Inhibition of wound surface flora and bacteria
Long shelf life, minimal storage requirements
Biodegradable (for permanent membranes)
Low cost
Minimize nursing care of wound
Minimize patient discomfort
Translucent properties to allow direct observation of healing
Reduce heal time
Not increase rate of infection
Patient acceptance
Stably enhance using nonviral vectors

* As previously identified by Pruitt and Levine and others.^{7,9,14}

Table 2

Patient Summary

Patient Number	Age (yr)/Gender	TBSA (%)	Cause of Injury
1	40/male	5	Motor vehicle (degloving trauma)
2	64/male	17.5	Motor vehicle (burn)
3	65/female	6	House fire (burn)
4	52/male	16	House fire (burn)
5	21/male	13	Campfire (burn)
6	52/female	8	Infection/surgical resection
7	46/female	15	Infection/surgical resection
8	29/male	73	Explosion (burn)
9	38/male	12	Explosion (burn)
10	66/male	25	Explosion (burn)
11	48/female	13	Clothing fire (burn)
12	20/male	17.5	Electrical (burn)
13	34/male	42.5	Paper mill explosion (burn)
14	38/male	31	Gasoline (burn)
15	38/male	38.5	Gasoline (burn)