Clinical Efficacy and Mechanism of Bilayered Living Human Skin Equivalent (HSE) in Treatment of Diabetic Foot Ulcers

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ABSTRACT

Bilayered living human skin equivalent (HSE) consists of cultured keratinocytes residing on the surface of a fibroblast-populated collagen lattice. Although HSE is FDA-approved for treatment of diabetic foot and venous stasis ulcers, its clinical efficacy remains limited, because the molecular mechanisms underlying its therapeutic effect are not fully understood. It is, therefore, often applied mistakenly as a skin graft. In this report, we delineate a mechanism of HSE biological effect and consequent optimal clinical use in accelerating closure of diabetic foot ulcers. *Experimental*: HSE was grafted onto nude mice and the release of various growth factors was evaluated by reverse transcription-polymerase chain reaction (RT-PCR) and immunochemistry. *Clinical*: HSE was grafted onto 11 consecutive patients with diabetes who had 13 non-ischemic foot ulcers, and healing was measured as time to 100% closure (e.g., no drainage and 100%

epithelialization). *Experimental*: HSE cellular components were determined to express 15 different growth factors/cytokine genes known to promote wound healing. Histological evidence from the nude mice showed that the collagen component of HSE underwent remodeling within the first seven days of grafting. *Clinical*: All diabetic foot ulcers healed in 31.8 ± 12.4 days. Local release of a unique combination of 15 growth factors expressed by HSE keratinocyte and fibroblast components generates closure of diabetic foot ulcers. HSE should be applied with the same surgical conditions necessary for a skin graft (i.e., no cellulitis, no drainage, and negligible bacteria). We hypothesize that bilayered HSE generates its effect by way of the local synthesis and release of multiple growth factors in specific combination and concentration, which improves the impaired reparative process of chronic wounds.

INTRODUCTION

Diabetic foot ulcers affect approximately 600,000 people each year and are the leading cause of hospitalization for patients with diabetes. Of the estimated 150 million patients with diabetes in the world,[1] 22.5 million can be expected to develop foot ulcers at some stage of their life. The extent of morbidities associated with these ulcers approach epidemic proportions. In the United States (U.S.), 162,500 patients with diabetes are hospitalized for foot ulcers annually.[2] The diabetic population in the U.S. undergoes 86,000 diabetes-related, lower-extremity amputations per year,[3] 84% of which are preceded by a foot ulcer.[4] Furthermore, amputations affect patients with Type 1 diabetes at a similarly high rate as patients with Type 2 diabetes.[5] Diabetes-induced limb amputation has a five-year mortality rate of 39%-68%, and is associated with an increased risk of additional amputations in the same patients.[2] Three FDA-approved biological products for efficacy of wound healing are available on the market [e.g., platelet-derived growth factor (PDGF)-BB,[6,7] dermal equivalent,[8] and bilayered human skin equivalent (HSE)[9]]. Despite this, many diabetic foot ulcers do not heal and result in amputation, in part, because: 1) the mechanisms of these biological products as well as the pathogeneses of these ulcers are not yet fully understood, 2) these biological products are not widely used for patients whose wounds are not healing, and 3) they are not applied properly.

Wound healing is a complex and organized regenerative biological and cellular response to tissue injury. Acute wounds heal in a timely and orderly process of cell replication, migration, protein synthesis, matrix deposition, and organization that results in sustained restoration of cellular activity, as well as tissue integrity and function. Diabetic foot ulcers, however, are chronic wounds that, by definition, fail to heal in a regulated and systematic manner. This failure is often due to an underlying physiologic impairment (e.g., decreased angiogenic response, neuropathy, ischemia) that compromises anatomic and functional integrity.[10] In particular, diabetic foot ulcers can generate endothelial dysfunction[11,12] and neuropathy;[13] on a cellular level, these wounds are characterized by a decrease in fibroblast proliferative capacity,[14] as well as abnormalities in extracellular matrix and cellular infiltrate.[15] Additionally, experimental diabetic wounds are characterized by decreased angiogenesis.[16]

Patients with diabetes exhibit impaired wound healing as well as increased susceptibility to wound infection.[17] Conse-

Table 1. Local Molecular Growth Factor and Cellular Abnormalities in a Diabetic Wound

1.	Growth-factor and cytokine abnormalities in wounds of diabetic mouse models and human diabetic wounds: VEGF,[21] IGF-I,[22,23] IGF-II,[23] TGF-β,[24] αFGF,[25] IL-6,[26] KGF.[25]
2.	Endothelial dysfunction.[11,12]
3.	Neuropathy is associated with endothelium-dependent and -independent dysfunction in diabetic patients predisposed to foot ulceration.[13]
4.	Decreased angiogenesis in experimental diabetic wounds.[16]
5.	Abnormalities in fibroblast function, decrease in fibroblast proliferative capacity.[14]
6.	Abnormalities in extracellular matrix and cellular infiltrate.[15]

VEGF = vascular endothelial growth factor
IGF = insulin-like growth factor
TGF = transforming growth factor
FGF = fibroblast growth factor
IL = interleukin
KGF = keratinocyte growth factor

Table 2. Primer Sequences for Control, Cytokines, and Growth Factors

GM-CSF	3' ctggctcccagcagtcaaaggg	5', atgtggctgcagagcctgctgc
G3PDH	3', catgtgggccatgaggtccaccac	5', tgaaggtcggagtcaacggatttggt
IGF-II	3', tgcggcagttttgctcacttccgattgctgg	5', agtcgatgctggtgcttctcaccttcttggc
IL-1α	3', tagtgccgtgagtttcccagaagaagaggagg	5', aaggagagcatggtggtagtagcaaccaacg
IL-6	3', gaagagccctcaggctggactg	5', atgaactccttctccacaagcgc
IGF-1	3', ccctctacttgcgttcttcaaatgtacttcc	5', acatctcccatctctctggatttccttttgc
PDGF-A	3', ctgcttcaccga gtgctacaatacttgct	5', agaagtccaggtgaggttagaggagcat
PDGF-B	3', gccgtcttgtcatgcgtgtgcttgaatttccg	5, ctgtccaggtgagaaagatcgagattgtgcgg
TGF-α	3', ggcctgcttcttctggctggca	5', atggtcccctcggctggacag
TGF-β1	3', aggctccaaatgtaggggcagg	5', gccctggacaccaactattgct
TNF-α	3', gcaatgatcccaaagtagacctgcccagact	5', gagtgacaagcctgtagcccatgttgtagca

GM-CSF = granulocyte macrophage-colony stimulating factor
G3PHD = glycerol-3-phosphate dehydrogenase
IGF = insulin-like growth factor
IL = interleukin
PDGF = platelet-derived growth factor
TGF = transforming growth factor

quently, if disruption of the integument in a patient with diabetes is not recognized and treated early, the patient is at risk of developing complications (e.g., bacterial colonization of the wound bed, soft tissues, bone, and/or bloodstream) associated with chronic wounds. Furthermore, chronic wounds that become infected can result in limb amputation. For these reasons, early intervention is crucial for successful treatment and averting the morbidity and mortality with which these ulcers are so closely associated.[18] Successful intervention, in turn, requires a standardized, effective, and rapid treatment protocol.

HSE is a bilayered, biologically active skin construct, composed of a surface layer of allogeneic human keratinocytes over a layer of allogeneic human fibroblasts, suspended within a bovine tendon collagen matrix. Bilayered living HSE is reported to have a 56% success rate in healing diabetic foot ulcers,[9] and its use proved effective in treating refractory venous stasis ulcers,[19] pressure ulcers,[20] and other chronic wounds. However, the mechanism through which bilayered living HSE exerts its effects is not fully understood, which necessitates further study to optimize its efficacy of application.

The presence of endogenous growth factors, such as vascular endothelial growth factor (VEGF),[21] insulin-like growth factor (IGF)-I,[22,23] IGF-II,[23] transforming growth factor-Beta (TGF-β),[24] keratinocyte growth factor (KGF),[25] and interleukin (IL)-6,[26] is noticeably decreased in diabetic wounds (Table 1).[21-26] Therefore, we hypothesize that bilayered HSE may have its effect by way of the local synthesis and release of multiple growth factors in specific combination and concentration, which improves the impaired reparative process of chronic wounds. To test our hypothesis, the expression of growth factors in HSE was analyzed, both *in vitro* and *in vivo*, using reverse transcription-polymerase chain reaction (RT-PCR) and immunochemistry. Additionally, the laboratory findings were translated in the clinic and the efficacy of HSE in treatment of patients with diabetic foot ulcers was tested.

Furthermore, a common misconception remains that diabetic ulcers do not require intervention until the wound has remained open for an extended period of time. Such an approach to treatment can expose the patient to significant morbidity, including infection and amputation. The same principles and mechanism apply to pressure ulcers; i.e., early treatment should be initiated to halt progression. This study demonstrates that early treatment with bilayered HSE may prevent these complications from chronic wounds; e.g., diabetic foot ulcers and pressure ulcers.

METHODS

Semiquantitative RT-PCR Analysis and Protein Detection

Total ribonucleic acid (RNA) was extracted from the homogenized bilayered HSE samples using the Qiagen (Valencia, California, USA) Shredder/Rneasy® ex-

Table 3. Primer Sequences for the Fibroblast Growth Factors (FGF) and Extracellular Matrix Proteins

Fibronectin	3', agggaccacttctctgggagg	5', ccgggttctgagtacacagtc
FGF-1	3', atggctgaaggggaaatcaccac	5', cagaagagactggcaggggggag
FGF-2	3', tcagctcttagcagacattggaagaaaaag	5', ggagtgtgtgctaaccgttacctggctatg
FGF-7	3', ttgccataggaagaaagtgggctg	5', ctttgctctacagatcatgctttc

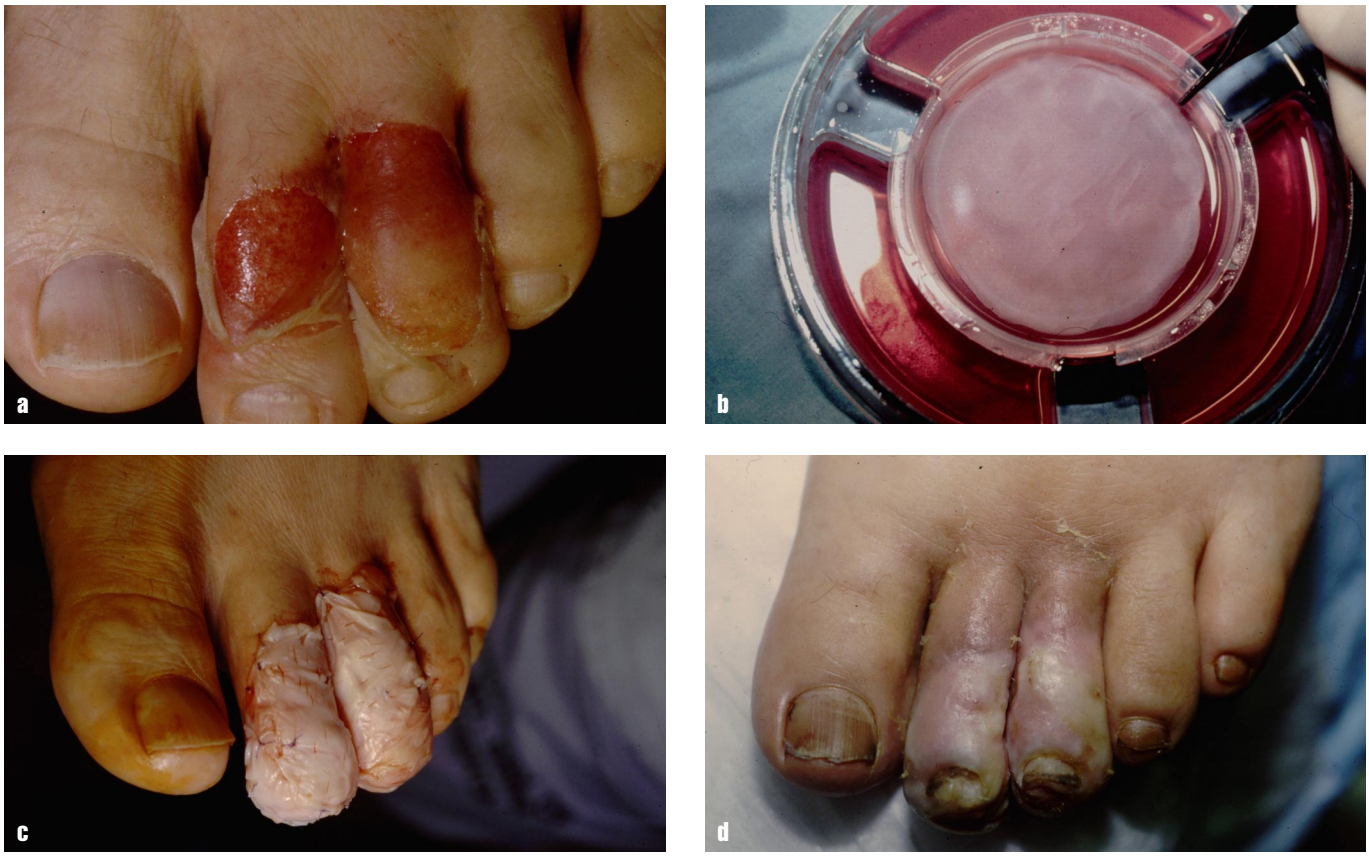


Figure 1. Healing of a diabetic toe ulcer. 1a: Diabetic patient with toe ulcers before treatment with bilayered living human skin equivalent (HSE). 1b: HSE, as it arrives in transwell, immediately before application. 1c: Same toe ulcers, immediately following application of HSE. 1d: Toe ulcers healed in 35 days.

traction protocol. Complementary deoxyribonucleic acid (cDNA) was synthesized following the standard protocol. Sequences of primers used in PCR are listed in Tables 2 and 3. For each primer, 5 L of cDNA from the aforementioned procedure were added to 31 L of diethylene pyrocarbonate-treated water, 5 L of 10x PCR buffer, 1 L of 10 mM of each nucleotide triphosphate (dNTP), 3 L of 25 mM $MgCl_2$, and 0.4 L of Amplitaq enzyme in thin-walled reaction tubes. After 35 cycles of amplification, the products were resolved on 2% agarose gel and photographed digitally using a change-coupled device camera. Intensities of band products were quantified using image analysis software (ImagePro®, Mediacybernetics, Inc., Silver Spring, Maryland, USA). Each of the different growth factors and cDNA solutions were corrected for various amounts of RNA by normalizing to a glycerol-3-phosphate dehydrogenase (G3PDH).

Enzyme-Linked Immunosorbent Assay (ELISA)

Commercial ELISA were used to determine the protein levels of IL-1 Alpha (IL-1α), IL-6, IL-8, IL-11, PDGF-AB, VEGF, granulocyte macrophage-colony stimulating factor (GM-CSF), and TGF-Beta1 (TGF-β1) (R&D Systems, Inc.,

Minneapolis, Minnesota, USA) following the manufacturer's instructions.

Animal Grafting

Permission for these animal experiments was obtained from the Institutional Animal Care and Use Committee. Athymic (*nu/nu*) mice (Jackson Laboratories, Bar Harbor, Maine, USA), 6-8 weeks in age and 24-32 Grams, were anesthetized using a mixture of ketamine and xylazine. A full-thickness 3-cm^2 excisional wound was created on the central dorsum of each mouse and a piece of HSE was grafted into the wound.[27] The graft was trimmed so that its edges abutted the surrounding skin edge. The graft was dressed with a piece of Vaseline®-impregnated gauze (Chesebrough-Ponds, Greenwich, Connecticut, USA). Two adhesive bandages were wrapped over the gauze and around the torso of the mouse. The dressing served to cover the wound and partially immobilize the mouse to protect the graft during the early post-graft period. After seven days, bandages were removed and the grafts were left uncovered for the duration of the experiment.

Histology

The entire full-thickness graft was excised, cut in half, and processed for either histology (formalin) or RT-PCR (snap frozen). Bilayered HSE grafts were biopsied at either four or seven days. These biopsies were fixed in 10% formalin and processed for hematoxylin and eosin (H&E), as well as Sirius red staining.[28] For Sirius red staining, sections were post-fixed for 24 hours in Bouin's solution. The post-fixed sections were stained for one hour in a saturated picric acid solution containing 1 mg/mL of picrosirius red, then washed in tap water, mounted with a coverslip, and viewed with an Olympus BH-2 microscope equipped with polarized light optics. Photographs were taken with Kodak Ektachrome™ (Eastman Kodak, Rochester, New York, USA) color slide film to record the collagen birefringence patterns.

CLINICAL STUDY DESIGN

Patients

Thirteen consecutive diabetic foot ulcers (6 mm-4.5 cm in diameter) in 11 patients were treated with HSE. Patients with evidence of osteomyelitis, as indicated by magnetic resonance imaging, were excluded from this study. The patients were treated immediately after their wounds were observed. HSE was applied only after the ulcers exhibited negligible drainage and the presence of well-vascularized granulation tissue (Figs. 1a-d). One patient, a multi-organ transplant recipient, was treated as an outpatient; our treatment protocol was instituted rapidly to prevent organ loss from potential wound infection. Seven of the 11 patients had plantar surface neuropathic foot ulcers. Approval for this retrospective chart analysis was obtained from the Institutional Review Board. None of these patients are reported in previously published manuscripts.

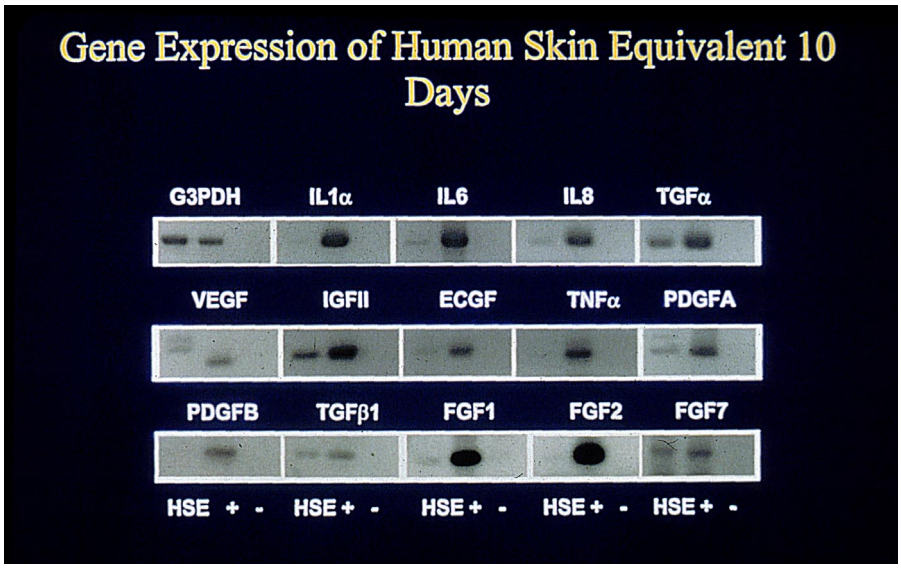


Figure 2. Gene expression of multiple growth factors in keratinocytes and fibroblasts in bilayered living human skin equivalent (HSE).

Surgical Technique of Applying HSE in Diabetic Foot Ulcers

The 11 patients in the study had 13 ulcers. Five of the 11 patients had cellulitis associated with diabetic foot ulcers and were treated with intravenous antibiotics in the hospital before application of HSE. Deep tissue samples were taken from the wound base for culture in all patients. Six of the 11 patients received only oral antibiotic coverage (appropriate for diabetic foot ulcers) until the deep tissue culture results were received. If no growth was present, the antibiotics were discontinued.

The wounds were prepared in the standard manner consistent with chronic wound care: 1) the wound base was excised, 2) the wound edges were extended by approximately 1 mm to 2 mm, and 3) a brisk capillary bleeding from all wound surfaces was attained. The goal of surgical debridement was to remove all necrotic and grossly infected material, non-viable tissue, and scar tissue. Adjacent hyperkeratotic tissue also was excised, even if this procedure required removal of the hyperkeratotic tissue from most of the plantar surface of the foot. The debridements were extended into the soft, non-hyperkeratotic, non-callous skin edge. Complete hemostasis was established, because blood can act as a medium for bacterial growth, and bacteria are toxic to cells.

After hemostasis was attained, HSE was applied with interrupted 5-0 absorbable sutures, with approximately 1 mm left between the HSE edge and new skin edge of the debrided wound. Diabetic foot ulcers are usually smaller than the prepackaged HSE portion—7.5 cm in diameter. For 12 of the 13 ulcers, the redundant HSE was placed over the entire wound with the fibroblast side down on the wound and keratinocyte side up. Two sutures placed outside the wound bed served to loosely secure the second layer of the HSE. Adaptic® (Johnson & Johnson, New Brunswick, New Jersey, USA) was placed over the graft, followed by Vaseline® gauze wrapped around sterile cotton or web roll, and then by Tegaderm™ (3M Health Care, St. Paul, Minnesota, USA). All patients were instructed to relieve pressure on the wound through off-loading. Off-loading modalities included crutches, orthotics, and occasional use of a wheel chair. No complications were observed in these patients.

Table 4. Cytokine and Growth-Factor Profile of Cell Populations Responsible for HSE

	MESSENGER RNA*			
CYTOKINE	Keratinocytes†	Fibroblasts†	HSE	PROTEIN HSE
IL-1α	Y	N	Y	Y‡
IL-6	N	Y	Y	Y‡
IL-8	Y	Y	Y	Y‡
IL-11	N	Y	Y	Y‡
IGF-I	N	N	Y	n.§
IGF-II	N	Y	Y	n.t.§
PDGF	Y	Y	Y	Y‡
TGF-α	Y	N	Y	n.t.§
VEGF	n.t.§	n.t.§	Y	Y‡
FGF-1	Y	Y	Y	n.t.§
FGF-2	N	Y	Y	n.t.§
FGF-7	N	Y	Y	n.t.§
GM-CSF	n.t.§	n.t.§	Y	Y‡
TGF-β1	N	Y	Y	Y‡
TGF-β3	N	Y	Y	n.t.§

*mRNA detected by reverse transcription polymerase chain reaction;
† Cells grown in monolayer cultures; ‡Protein detected by enzyme-linked immunosorbent assay; §n.t.= not tested

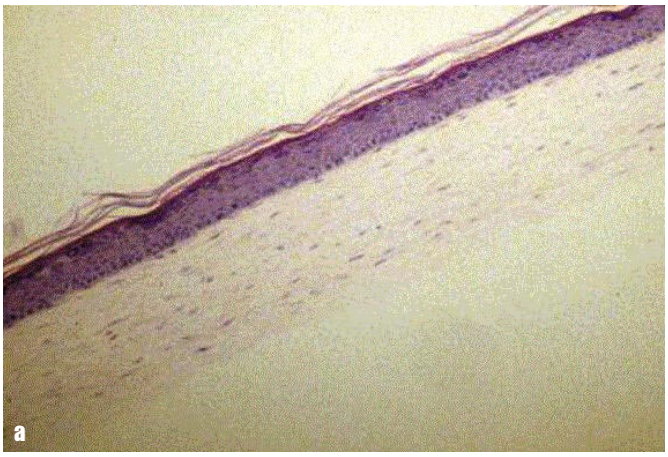
HSE = human skin equivalent
IL = interleukin
IGF-N = insulin-like growth factor
PDGF = platelet-derived growth factor
TGF = transforming growth factor
VEGF = vascular endothelial growth factor
FGF = fibroblast growth factor
GM-CSF = glycerol-3-phosphate dehydrogenase

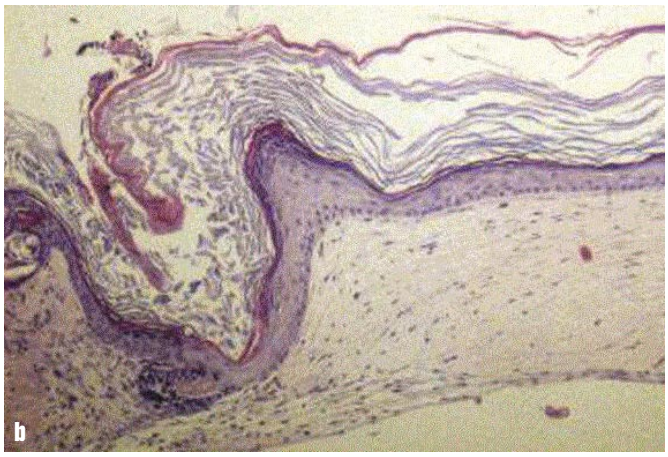
RESULTS

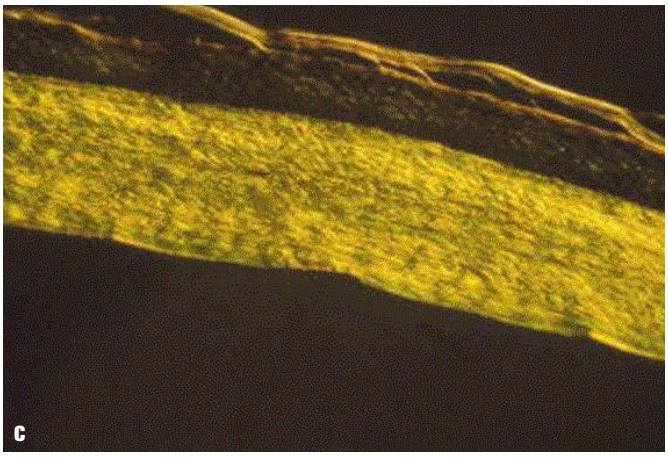
To identify the expression of growth factors and cytokines important for the wound-healing process, HSE was transplanted onto athymic mice. The expression and release of a select number of growth factors and cytokines were measured by RT-PCR and ELISA. Over the entire study period (17 days post-graft), HSE expressed 15 distinct growth factors/cytokines associated with wound healing, maintenance of normal skin function, or both (Fig. 2). In addition, time-dependent distribution of various factors was noted (data not shown). Specifically, the expression of pro-inflammatory cytokines, such as IL-1α and IL-6, peaked early (four hours post-graft). In contrast, other growth factors (cytokines: TGF-β, PDGF-A, IGF-II) peaked later, at 96 to 168 hours post-graft.

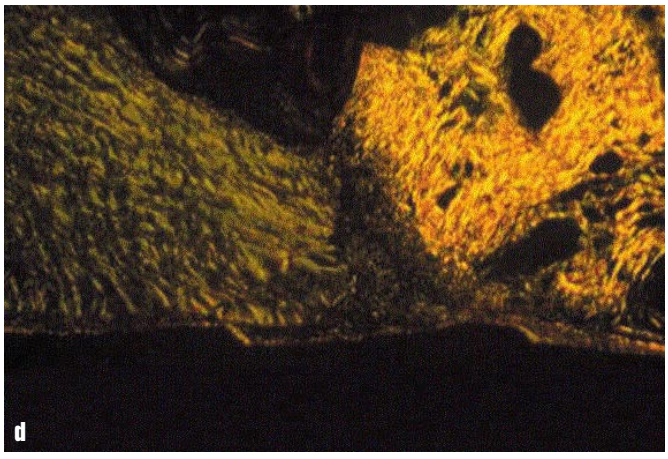
To further evaluate whether the protein production correlated with the gene-expression profile, HSE also was analyzed for cytokine protein production using ELISA (Table 4). Importantly, the growth factor/cytokine gene-expression profile did correlate with protein production in the HSE. Analysis from separately grown fibroblast and keratinocyte cell populations suggested that not only did both cell populations synthesize growth factors individually, but they also worked in synergy, thus creating the final cytokine profile (see Table 4). For example, neither cell population individually expressed the cytokine IGF-I in monolayer culture, but did so when incorporated within the HSE.

Histological characteristics of the HSE showed a moderate increase in fibroblast density, on average, from 18 (range: 17-20) fibroblasts before application of HSE (Fig. 3a), to 28 (range: 26-30) fibroblasts at four days post-grafting. The fibroblasts in the four-day-grafted HSE were oriented parallel to the surface, and disclosed a thick, viable layer of differentiating keratinocytes. The morphology of the keratinocytes growing on the surface remained unchanged on the grafted HSE at four and seven days, and they were firmly attached (Fig. 3b). The birefringence pattern of collagen fibers within the grafted HSE showed a moderate degree of organization with the collagen fibers running in parallel arrays (Fig. 3c). The birefringence pattern was altered in the four-day grafts, with a more random orientation of collagen fibers associated with a decrease in the birefringence









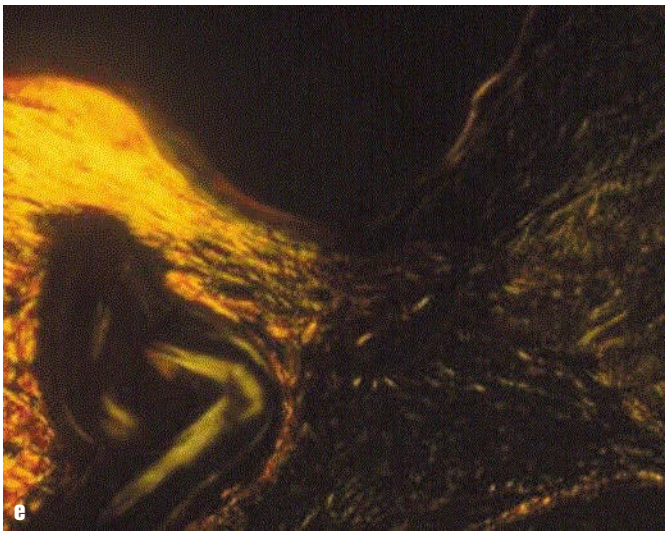


Figure 3. Histological analysis.
3a. Hematoxylin and eosin (H&E) stained human skin equivalent (HSE) before grafting on a mouse. [Magnification 30 times.]
3b. Graft at seven days shows junction between graft and dermis (on left). Note excellent take of HSE and intact epithelialization over junction. Fibroblast density within graft site is similar to that of intact HSE (see above). [Magnification 30 times.]
3c. Sirius red-stained polarized light viewed histology of HSE before grafting. Note pattern and degree of green birefringence that has occurred *in vitro,* indicating organization of collagen by resident fibroblasts.
3d. HSE graft four days after grafting, stained with Sirius red and viewed with polarized light. Normal dermis with its strong yellow-orange birefringence pattern on the right and HSE with its fine green birefringence pattern on the left. Comparing HSE birefringence pattern before implantation, it appears to have become weaker after four days post-graft. [Magnification 60 times.]
3e. Seven-day graft stained with Sirius red and viewed with polarized light, showing strong birefringence yellow-orange pattern of normal dermis on left and fine green birefringence pattern of HSE on right. Comparing HSE birefringence pattern at four days, it appears to have continued waning by seven days. [Magnification 60 times.]

intensity (Fig. 3d). The loss of birefringence intensity implies collagen underwent turnover within the graft. At seven days, intensity of the collagen birefringence was further reduced, which indicated that collagen turnover was continuing in the HSE graft (Fig. 3e).

Patient Studies

HSE was placed on 11 consecutive previously unreported patients with diabetes who had 13 non-ischemic surface foot ulcers. The ulcers had an average surface area of 4.47 cm^2 and were located on the toe, plantar surface, and/or dorsum of the foot. All treated wounds healed completely in 31.8 ± 12.4 days. In all 13 diabetic wounds treated, complete healing was observed in an average of 32 days with a single application of HSE.

DISCUSSION

This report demonstrates that non-ischemic diabetic foot ulcers without osteomyelitis can heal after application of bilayered living HSE. It was hypothesized, based on these findings, that HSE efficacy may result from the local release of a combination of 15 distinct growth factors and cytokines by the keratinocytes

and fibroblasts that comprise HSE, promoting wound closure. Many clinical and experimental reports describe the significant impact a single growth factor can have on wound healing.[29-33] The data herein emphasize that although the growth factors released by HSE may be present at a lower concentration when compared to a single purified growth-factor therapy, their synchronized presence may be sufficiently effective for promoting the closure of chronic wounds.

In chronic wounds that exhibit decreased growth-factor production, cell therapy and their subsequent release of growth factors presents one appropriate treatment option for healing and closure of these wounds. Cell therapy does not necessarily "replace" the impaired wound-healing response or deficient growth-factor production in a diabetic ulcer; rather, it is applied topically to the wound and is made up of fibroblasts and keratinocytes—two types of cells that release growth factors and must be present and active for normal healing of a wound. Keratinocytes secrete substances that control the cellular-activation cycle responsible for the wound-healing process. Keratinocytes at the wound edge release pre-stored interleukin-I, the first signal released at the time of initial tissue injury. This process activates other keratinocytes and the surrounding cells that begin the synthesis of additional growth factors and cytokines.[34-37] These signaling molecules have a chemotactic effect on white blood cells, and a paracrine effect on some of the major cells involved in a healing wound; e.g., fibroblasts and endothelial cells.

The findings herein suggest that the name, "human skin equivalent," is misleading because it implies the cells are equivalent to an autologous skin graft. This misunderstanding is exacerbated by the original intended use of bilayered living HSE as a skin substitute for burn patients, because its physical and biological features are similar to those of human skin—specifically, barrier function, matrix, and living cells. The differences between HSE and a skin graft must be delineated. Bilayered HSE is a cellular therapy that accelerates closure of chronic wounds, in part by supplying the cells that release growth factors that stimulate closure. A skin graft provides permanent coverage. No biological treatment is a replacement for plastic surgical techniques such as skin grafts, and these should continue to be made available to all patients who may benefit from them. One is not an alternative to another; they are both treatment options.

The patients in this study included the elderly, psychiatrically impaired, and/or immunosuppressed, and their wounds included heel ulcers and previously infected ulcers. All wounds healed rapidly. Our resulting data established that when bacteria is removed by debridement and other appropriate therapies, intervention with bilayered living HSE, or cell therapy, provides a treatment option that ensures accelerated wound closure. Every wound without ischemia or osteomyelitis can heal, but chronic wounds heal more slowly. The status of a wound should not be judged by its appearance. A wound can "look good," but nevertheless be a source of sepsis.

Growth-factor therapy alone may not effect wound healing.[32] Growth factor and cytokine therapies are designed to augment, not replace, a comprehensive wound-care regimen that includes off-loading, debridement, control of infection, and other treatments. We hypothesize that if all diabetic wounds are recognized early and treated promptly with a regimen that includes cell and/or factor therapy if the wound is not healing (as measured objectively by contraction and epithelialization), the incidence of osteomyelitis and amputation in non-ischemic wounds will decrease dramatically. We have noticed similar results for pressure ulcers[20,38] (e.g., sacral, ischial, trochanteric, and heel), as well as with venous stasis ulcers.[39]

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