Objective: To test the safety, efficacy, and immunological impact of a cultured allogeneic human skin equivalent (HSE) in the treatment of venous ulcers.

Design: Prospective, randomized study.

Setting: Multicenter study in the outpatient setting.

Intervention: Each patient with a venous ulcer received either compression therapy alone or compression therapy and treatment with HSE. The patients were evaluated for HSE safety, complete (100%) ulcer healing, time to wound closure, wound recurrence, and immune response to the HSE.

Outcome: The study was completed as planned in 293 randomized patients.

Results: Treatment with HSE was more effective than compression therapy in the percentage of patients healed by 6 months (63% vs 49%; P=.02, Fisher exact test, 2-tailed) and the median time to complete wound closure (61 days vs 181 days; P=.003, log-rank test). Treatment with HSE was superior to compression therapy in healing larger (>1000 mm²; P=.02) and deeper ulcers (P=.003) and ulcers of more than 6 months’ duration (P=.001). Occurrence of adverse events was similar in both groups. No symptoms or signs of rejection occurred in response to treatment with HSE, and no HSE-specific immune responses were detected in vitro to bovine collagen or to alloantigens expressed on keratinocytes or fibroblasts.

Conclusions: Treatment with HSE healed venous ulcers more rapidly and in more patients than compression therapy alone. There was no clinical or laboratory evidence of rejection or sensitization in response to HSE application. These data suggest that HSE represents a significant advance in the treatment of venous ulcers, particularly those that are difficult to heal.

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VENOUS ulcers are chronic wounds associated with long-standing venous hypertension of the lower extremities.1-4 The number of individuals affected by these ulcers in the United States is greater than 600,000, a figure likely to be an underestimate in view of our increasing elderly population.5 While venous ulcers are not a cause of limb loss, they are a major cause of morbidity, and their care is costly. They necessitate frequent visits to physicians and by visiting nurses, cause loss of productivity in the young and increased frailty in the elderly, require patients to deal with bulky and malodorous dressings, and commonly lead to hospitalization for often life-threatening cellulitis. The impact of these and other complications of venous ulcers on overall health and quality of life is only beginning to be appreciated.6

Since Unna, a 19th-century dermatologist, devised an early zinc oxide–impregnated compression bandage for the treatment of venous ulcers, the main therapy for these wounds has been compression of the affected limb.5-7-10 Elastic and nonelastic compression bandages are made and applied in a variety of ways, but no substantive advances have been made in the way venous ulcers are treated. Autologous split-thickness grafting and surgical reconstruction to correct or improve venous abnormalities often succeed where conservative therapy with compression bandages fails,11,12 but these proce-
PATIENTS, MATERIALS, AND METHODS

MATERIALS

The composition of HSE, previously referred to as a living skin equivalent, has been described in detail elsewhere. Briefly, HSE is a bilayered skin product consisting of a dermal equivalent composed of type I bovine collagen that contains living human dermal fibroblasts and an overlying cornified epidermal layer of living human keratinocytes. The fibroblasts and keratinocytes of HSE (used within their first 2 to 3 in vitro passages) are derived from neonatal foreskin that has undergone extensive safety testing, including rigorous screening for a panel of known adventitious agents such as human immunodeficiency virus and hepatitis viruses. A schematic representation of how the HSE is prepared is shown in Figure 1, while Figure 2 shows a photomicrograph of a cross section of HSE, with normal human skin for comparison. The HSE was delivered to study centers in a ready-to-use form on nutrient agarose in a sealed bag and gassed with 10% carbon dioxide. Immediately after being received, the HSE was kept in a 37°C incubator until use, which was within 5 days of delivery.

STUDY DESIGN

The study was a prospective, 15-center, controlled, parallel-group, comparative trial. Eligible patients aged 18 to 85 years were randomly assigned in the outpatient setting to receive either control treatment, consisting of compression therapy alone, or HSE immobilized with an elastic compression bandage. Patients were followed up for 12 months. For analysis of safety and efficacy, end points were prospectively set at 6 months, with an additional 6 months for further safety evaluation. Patients were entered into the study after informed consent was obtained. Patients who qualified to participate in the study were assigned to either the HSE or control treatment groups according to computer-generated randomization schedules held by the sponsor and the contracted data management group.

STUDY POPULATION

In total, 309 patients were randomized for the trial and 293 were treated; for final statistical analysis, 275 patients who met inclusion and exclusion criteria were evaluated, with 129 patients randomized to compression therapy alone (control) and 146 to HSE treatment. The ulcers were due to venous insufficiency as determined by the following criteria: (1) presence of clinical signs and symptoms of venous ulceration, such as hyperpigmentation of the surrounding skin, varicosities, and lipodermatosclerosis; (2) absence of significant arterial insufficiency (as determined by an ankle brachial index >0.65); and (3) evidence of venous insufficiency by air plethysmography or photoplethysmography; with venous refill time being less than 20 seconds. Exclusion criteria were as follows: clinical signs of cellulitis, vasculitis, or collagen vascular diseases, pregnancy or lactation, uncontrolled diabetes mellitus, and other clinically significant medical conditions that would impair wound healing, inclusive of renal, hepatic, hematologic, neurologic, or immunologic disease. Patients receiving corticosteroids, immunosuppressive agents, radiation therapy, or chemotherapy within 1 month prior to entry into the study were also excluded. Before inclusion into the study, the ulcer had to be free of cellulitis and exudation indicative of heavy bacterial contamination and could not contain an eschar or obvious necrotic material that would interfere with graft take and healing.

TREATMENT PROTOCOL AND FOLLOW-UP

The ulcer sites of the control patients were dressed with a nonadherent primary dressing (Tegapore, 3M Health Care, St Paul, Minn) gauze bolster, zinc oxide–impregnated past bandage (Unna boot), and self-adherent elastic wrap (Coban, 3M Health Care). In the HSE treatment group, the HSE was placed directly on the ulcer; the nonadherent primary dressing was applied, followed by the cotton gauze dressing folded as a bolster; the same elastic wrap (Coban) used in control patients was used to immobilize the HSE and to keep it in place. Application of the HSE to a venous ulcer is shown in Figure 3. The elastic wrap, which extended from the metatarsal heads to the infrapatellar notch, was placed at midstretch tension with at least 50% overlap in both treatment groups. The HSE could be applied up to a maximum of 5 times during the initial 3 weeks of the study (on visit days 0, 3-5, 7, 14, and/or 21). If, by clinical observation, the “percent take” of HSE (coverage apparent by clinical inspection) was less than 50%, another application of HSE to the ulcer site was required. Ulcer sites with percent take estimated at greater than 50% were not permitted another application. No patient received more than 5 HSE applications, and no HSE was applied after week 3 of the study. In the control group, compression therapy alone was reapplied weekly for the first 8 weeks. The frequency of subsequent dressing changes was the same for both treatment groups. Thus, after 8 weeks or on complete healing (defined as full epithelialization of the wound and no drainage from the site), patients in both treatment groups used...
graded elastic stockings as a means of compression for the remainder of the study. If ulcers were not completely healed or if excessive drainage did not permit use of elastic stockings, patients continued receiving wound dressing treatments according to the group to which they had been randomized.

STUDY EVALUATIONS

All patients were evaluated weekly for the first 8 weeks. After 8 weeks, they were evaluated at 12 weeks and every 3 months thereafter for up to 12 months. At each visit, ulcer healing was evaluated and recorded by photographs and wound tracings. Ulcer size was determined by computerized planimetry of surface tracings made with a plastic film. The study was prospectively defined as being completed at 6 months for statistical analysis of safety and effectiveness. An additional 6-month follow-up period was defined for further safety evaluation. The primary efficacy endpoints included incidence of complete healing by 6 months after initiation of treatment and the time required for complete healing to occur. Other efficacy endpoints evaluated were incidence and time to 50% and 75% wound closure by 6 months. Safety was evaluated by several parameters, including spontaneous reports of adverse events at each visit and laboratory evaluations (serum chemistry profile and complete blood cell count) on day 0 and at month 6.

IMMUNOLOGICAL TESTS

Sample Collection

Serum and heparinized whole blood samples were collected from each patient at baseline, week 1, week 4, and month 6. Peripheral blood mononuclear cells were isolated from whole blood samples by density gradient centrifugation over a lymphocyte separation medium (Organon Teknika, Durham, NC). Keratinocytes (2 × 10⁵ per well), as previously described, were cultured in 96-well plates (Corning Costar, Kennebunk, Me) with pooled allogeneic peripheral blood mononuclear cells (1 × 10⁹ per well), as previously described. Keratinocytes (2 × 10⁶ per well) or fibroblasts (2 × 10⁶ per well) were used as a source of alloantigen. Stimulator keratinocytes and fibroblasts were treated with interferon gamma to induce expression of HLA class II antigen. After 5 days, cultures were pulsed with 0.037 MBq of tritiated thymidine and harvested the next day. Response was defined as the difference in tritiated thymidine uptake between stimulated cells and unstimulated controls.

Detection of Allo-Specific T Cells

Patient peripheral blood mononuclear cells (1 × 10⁵ per well) were cultured in 96-well plates (Corning Costar, Kennebunk, Me) with pooled allogeneic peripheral blood mononuclear cells (1 × 10⁹ per well), as previously described. Keratinocytes (2 × 10⁶ per well) or fibroblasts (2 × 10⁶ per well) were used as a source of alloantigen. Patient peripheral blood mononuclear cells (1 × 10⁵ per well) were cultured with pooled allogeneic peripheral blood mononuclear cells (1 × 10⁹ per well), as previously described. Keratinocytes (2 × 10⁶ per well) or fibroblasts (2 × 10⁶ per well) were used as a source of alloantigen. Stimulator keratinocytes and fibroblasts were treated with interferon gamma to induce expression of HLA class II antigen. After 5 days, cultures were pulsed with 0.037 MBq of tritiated thymidine and harvested the next day. Response was defined as the difference in tritiated thymidine uptake between stimulated cells and unstimulated controls.

Detection of HLA-Cytolytic Antibodies

An assay for complement-dependent microlymphocytotoxicity against HLA class I antigens was performed using an assay kit (Lambda Cell Tray, One Lambda Laboratories, Canoga Park, Calif) according to the manufacturer’s instructions. Pooled human antibody-negative serum was run simultaneously as a negative control for lymphocyte viability. A serum sample was scored as positive if it specifically lysed cells (<20% cell viability) to the HSE.

STATISTICAL ANALYSIS

All statistical calculations were performed using SAS software (version 6.10 for Windows, SAS Inc, Cary, NC). Comparisons between treatment groups for demographics and baseline ulcer evaluations were performed using the Fisher exact test or χ² analysis. In addition, an analysis of frequency of wound closure was determined by the Fisher exact test (2-tailed), and time to wound closure was computed using a survival analysis by the Kaplan-Meier life-table method. Statistical significance of the difference in time to wound closure by treatment was tested using the log-rank test. The Fisher exact test was used to evaluate incidence of wound recurrence. Because previous reports have cited indicators of success or failure in the treatment of patients with venous ulcers, the group of clinical investigators involved in this study identified several preexisting factors thought likely to affect outcome. Therefore, it was prospectively decided to perform Cox proportional hazards regression analysis on the following variables: size, depth, and duration of the ulcer. The individual and combined effects of these factors on time to wound closure were analyzed.
RESULTS

There were no significant differences between the control and HSE treatment groups in patient demographics and baseline ulcer size and duration (Table 1). The typical appearance of HSE when applied to a venous ulcer is shown in Figure 4. The HSE was easy to handle and to apply to the ulcer, and its application was not associated with any pain or discomfort. Treatment with HSE proved more effective than compression therapy alone in a number of key parameters. When compared with control treatment at the 6-month time point, HSE treatment reduced by 3-fold the median time to complete healing (P = .003; Table 2). This accelerated healing observed in ulcers treated with HSE was consistently present throughout the treatment period, as shown by statistically significant decreases (P = .02 and P = .001, respectively) in the days needed for 50% and 75% reduction in ulcer area (Table 2). Moreover, as shown in Table 2, the proportion of patients with complete wound closure was significantly greater for patients treated with HSE (P = .02). The greater effectiveness of HSE treatment over control treatment was also evident on survival analysis by the Kaplan-Meier life-table method (Figure 5). As determined by Cox proportional hazard regression analysis, the average patient treated with HSE had a 54% better chance for wound closure per unit time than a patient in the control group (P < .001; 95% confidence interval, 1.275-1.855). The greater effectiveness of HSE therapy compared with compression therapy was also supported by data from the intent-to-treat group. The average number of HSE applications for each patient was 3.34. Treatment differences were uniform across study centers, favoring HSE over control.

These data indicate that this allogeneic cultured HSE may represent an important advance in the treatment of venous ulcers. Next, we determined whether the effectiveness of this novel treatment extended to difficult-to-heal ulcers. When subjects were stratified according to baseline characteristics, important differences between the efficacy profiles of HSE and compression therapies became evident. In the subgroup of subjects with ulcers of greater than 6 months' duration, the HSE-treated pa-

Figure 1. Schematic and simplified representation of how the human skin equivalent is prepared. Human neonatal fibroblasts and type I bovine collagen are allowed to form a gel, on top of which human neonatal keratinocytes are subsequently added. Exposure of the keratinocyte layer to the air-liquid interphase allows keratinocytes to differentiate and form a horny layer.

Figure 2. Photomicrographs of section through the human skin equivalent (A) as compared with normal human skin (B).
Patients healed significantly faster than the control-treated patients (median of 92 days vs 190 days to complete healing, respectively; \(P=.001\), log-rank test). However, in subjects with ulcers of less than 6 months’ duration, HSE treatment was as effective as compression therapy (46 days to closure vs 89 days, \(P>.05\)). This finding suggests that HSE may have certain biologic and physical characteristics that make it particularly useful in difficult-to-heal ulcers. Similarly, in the subgroup of patients with stage III ulcers (down to muscle), HSE promoted a shorter time to closure than was found in control patients (83 days vs 183 days; \(P=.003\), log-rank test).

We fully realize that it is not customary to classify venous ulcers by depth using the classification developed for pressure ulcers. For example, most venous ulcers extend down to the dermis; moreover, it would be highly unusual for venous ulcers to extend down to bone. However, the investigators were basically asked to establish whether they were dealing with a superficial ulcer (stage II) or a deeper ulcer extending to the subcutaneous tissues (stage III). Therefore, the data indicate that HSE is able to heal deep ulcers. Again, HSE was as effective as compression therapy in treating stage II ulcers (57 days vs 98 days; \(P=.05\), log-rank test). Finally, in both subgroups of patients with large ulcers (>1000 mm\(^2\)) or small ulcers (<1000 mm\(^2\)), treatment with HSE promoted faster closure than compression therapy (181 days vs 231 days, large ulcer, \(P=.02\); 56 days vs 98 days, small ulcer, \(P=.04\)).

The process by which healing occurred in the HSE-treated group is not entirely clear and will need to be addressed in future studies specifically designed to answer
this important question. However, from clinical observations made by the investigators and recorded in the case report forms, the following can be stated. Frank “graft take” and at least temporary persistence of the HSE were observed in 41% of the patients who healed with HSE treatment, but remodeling of the graft and probable replacement with the patient’s own skin appeared to occur in at least 63% of these patients. Conversely, healing by secondary intention, with no obvious persistence of the graft, was observed in 59% of the patients who healed with HSE treatment. It is likely that these observations reflect different ways by which HSE can benefit these wounds, either as a temporary skin replacement or as a stimulus for wound healing.

Venous ulcer recurrence is a major clinical problem; all patients with healed ulcers were therefore followed up for evaluation of the recurrence rate associated with HSE treatment. It should be noted that earlier closure of HSE-treated ulcers resulted in a longer period of time for assessment of wound recurrence in the HSE-treated group compared with the control-treated group. However, wound recurrence rates after complete healing of the ulcers still did not differ significantly between the 2 groups during the 12-month study period. Ulcers recurred in 10 (15.9%) of 63 patients who had been treated with compression therapy alone and in 11 (12%) of the 92 patients who had received HSE treatment ($P=0.48$, 2-tailed Fisher exact test).

There were no clinical signs of rejection of the HSE during the study. Specifically, use of the HSE was not associated with increased pain, erythema, other local or systemic inflammatory signs, or visible signs of necrosis. Also, there was no evidence of tissue breakdown within the ulcers after healing or, as previously stated, increase in ulcer recurrence. Laboratory results (see below) confirmed our clinical observation of the lack of rejection.

### IMMUNOLOGICAL TESTING

At baseline, none of the control-treated ($n=122$) or HSE-treated ($n=147$) patients tested showed evidence of pre-existing cytotoxic antibodies specific for the HLA class I antigens expressed on HSE cells. Retesting done at week 1, week 4, and month 6 after the application of HSE showed that patients treated with HSE do not develop such antibodies (Table 3). While T cells from HSE-treated patients proliferated in response to alloantigens presented by pooled allogeneic peripheral blood mononuclear cells, patients’ T cells did not proliferate in response to class II antigens present on allogeneic keratinocytes or fibroblasts (Figure 6). No significant

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**Figure 5.** Proportion of randomized (intent-to-treat) patients achieving complete wound closure in the 2 groups treated with control therapy or with human skin equivalent (HSE).

**Figure 6.** In vitro assay for sensitization of the cellular immune response by human skin equivalent (HSE). Human epidermal keratinocytes and human dermal fibroblasts from the same donor as cells used to make HSE did not stimulate proliferation of T cells from HSE-treated patients. dCPM indicates counts per minute.

**Table 3.** Number of Patient Serum Samples Containing Anti-HLA Cytotoxic Antibody or Anti–Bovine Type I Collagen Antibody Titers

*HSE indicates human skin equivalent.

†The number of patients who completed the 6-month visit were 108 in the HSE treatment group and 85 in the control treatment group. The lower numbers of total patients tested reflect failure to obtain 6-month serum samples from some patients.
differences occurred in the distribution of positive and negative proliferative responses to keratinocytes and fibroblasts between control- and HSE-treated populations. In addition, no significant changes in response were seen from baseline to any time point for any of the stimuli (P > .05, McNemar exact test). Like the antibody results, the mixed lymphocyte culture results suggest that exposure to HSE did not induce the generation of allospecific T cells. All HSE-treated patients whose baseline anti–bovine collagen type I antibody titers were lower than 40 (negative) remained negative during the study (Table 3). Six patients (4 in the control treatment group and 2 in the HSE treatment group) had positive antibody titers at baseline; of the positive baseline titers in the 2 HSE-treated patients, 1 remained unchanged during the study and 1 became negative after treatment.

SAFETY

Adverse events attributable to treatment were similar in the 2 groups. There were no significant differences between HSE and control treatment groups in the frequency of wound infection, cellulitis, or pain, which were the 3 most commonly reported adverse effects; eg, cellulitis occurred around the ulcer in 12 patients in the HSE treatment group and in 10 patients in the control treatment group. Systemic antibiotics were used to treat this complication. However, no effect on ultimate outcome was noted that could be attributed to the use of antibiotics. There was no statistically significant difference in the number of wound infections attributed to the 2 treatment groups. Reasons for dropout in the HSE treatment group were, in order of frequency, unavailable for follow-up (n = 12), death (n = 5), noncompliance or protocol violation (n = 5), adverse experience (n = 3), patient request (n = 2), and intercurrent illness (n = 2). In the control treatment group, the reasons for dropout were unavailable for follow-up (n = 9), patient request (n = 7), adverse experience (n = 7), noncompliance or protocol violation (n = 5), death (n = 4), and intercurrent illness (n = 1). No significant differences were found between the 2 groups in dropout rates or reasons for discontinuation of treatment.

COMMENT

In this randomized, prospective, comparative study of venous ulcer therapy in the outpatient setting, a cultured human allogeneic HSE consisting of both epidermal and dermal cells was shown to provide complete wound closure in a third of the time of compression therapy alone, without evidence of rejection or sensitization of patients to HSE antigens. The HSE-treated subjects enjoyed a greater number of wound-free days than the control-treated subjects, and HSE was particularly effective in difficult-to-heal ulcers, including those that were large, deep, or of long duration. The frequency of ulcer recurrence and the number of adverse events did not differ between control and HSE groups. The results of this study indicate that HSE represents an effective treatment for venous ulcers.

In this study, the first large, multicentered clinical trial reporting the use of an allogeneic bilayered skin equivalent, no clinical evidence of rejection or immune sensitization to HSE was observed in any treated subject. Specifically, there was no increase in pain, erythema, or tenderness, and no uplifting or deterioration of the HSE was noted after its application or after healing occurred. Laboratory results also showed no evidence of immunological reaction to alloantigens present on HSE keratinocytes or fibroblasts or to bovine collagen in patients treated with HSE. These findings suggest that, when used to treat venous ulcers, the HSE does not trigger an immunological response, since none of the HSE-treated patients were tissue matched or received immunosuppressive therapy. Perhaps the lack of immunological response is not surprising. The HSE used in this study does not contain professional antigen-presenting cells, which are necessary for activation of allogeneic T cells and tissue rejection. Professional antigen-presenting cells of the skin include Langerhans cells, dermal dendritic cells, endothelial cells, and passenger leukocytes. Fibroblasts and keratinocytes are nonprofessional antigen-presenting cells and do not activate unprimed allogeneic T cells, because they do not express HLA class II and common costimulatory molecules, such as B-7, CD-40, and intercellular adhesion molecule (ICAM). In addition, other lines of evidence indicate that HSE components could be safely used in allogeneic recipients. Type I collagen, as used in HSE, is composed of heterotrimeric molecules containing 2 α1 (I) chains and 1 α2 (I) chain. Type I collagen is weakly immunogenic, probably because collagen α chains show little interspecies amino acid variability.

Prior reports have already suggested the usefulness of components of living skin in the treatment of acute and chronic wounds. A cultured allogeneic skin equivalent, composed of a layer of autologous keratinocytes over allogeneic fibroblasts embedded in a noncontracted gel of animal collagen, accelerated healing in patients with leg ulcers. Cultured keratinocytes, without fibroblasts or a dermal component, have been used to successfully treat chronic leg ulcers and pressure ulcers in a number of small trials. It has been suggested in these earlier studies that allogeneic keratinocytes may survive in the wound only temporarily; the recipient’s cells appear to eventually replace the transplanted keratinocytes. Evidence for this phenomenon comes from DNA fingerprinting analysis and from the observation that ulcers treated with keratinocyte sheets heal from the edge, perhaps in response to stimulation by the transplanted cells. Indeed, substantial clinical evidence suggests that even autologous split-thickness grafts may act not only as replacement tissue, but also as a biologic agent capable of pharmacologic actions. Treatment with HSE may accelerate healing of venous ulcers by 3 mechanisms: stimulation of healing by HSE cellular and matrix components, with epithelialization proceeding from the edge of the wound and from islands of epithelium in the wound bed; biologic effects of occlusion, with stimulation of new skin tissue formation; and frank graft take with vascularization, integration, and eventual remodeling over time.
lated the healing of the ulcers through mechanisms other than tissue replacement.

In summary, HSE application in patients with venous ulcers was not accompanied by any laboratory or clinical signs of rejection or immunological sensitization. It accelerated the healing of difficult-to-heal venous ulcers and healed a significantly greater proportion of patients than compression therapy alone. Taken together, these results suggest that HSE may provide a highly effective treatment for these chronic wounds.

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