

Cultured Allogeneic Skin Cells are Effective in the Treatment of Chronic Diabetic Leg and Foot Ulcers

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Diabetic ulcers on the lower extremities present a difficult treatment problem, and some ulcers respond poorly to conventional topical and cast treatment. The purpose of this study was to assess the effect of cultured allogeneic keratinocyte epithelium and fibroblast-gelatin sponge on the healing of chronic, refractory diabetic leg and foot ulcers. Non-diabetic chronic leg ulcers were treated for comparison. This open study comprised 22 patients with type I or type II diabetes and 16 patients with leg or ankle ulcers of different aetiologies. A total of 26 diabetic and 25 non-diabetic ulcers were treated mainly with keratinocyte epithelium and/or fibroblast-gelatin sponge once weekly until complete healing or until no further healing could be observed despite several repeated treatments. The duration of diabetic ulcers was 10.3 ± 15.8 (mean \pm SD) months and the size 3.1 ± 6.6 cm². The diabetic ulcers were located in the heel (7), toe (7), sole (5), leg (6) and Achilles (1). The mean duration of non-diabetic ulcers was 6.8 ± 6.0 months and the size 10.5 ± 11.8 cm². A total of 12 ± 11 skin cell transplantations were performed for the diabetic ulcers. All but 1 diabetic ulcer healed during the study. The time for 50% reduction in ulcer area was 32 ± 32 days, but 99 ± 110 days were needed for complete ulcer closure. The longer the ulcer had existed the longer was the healing time. Heel ulcers showed significantly slower healing response than leg, sole and toe ulcers. Preliminary results suggest that both keratinocytes and fibroblasts are equally effective in the healing process. The time required for healing of the diabetic ulcers did not differ markedly from that of the non-diabetic ulcers. The results suggest that cultured allogeneic skin cells used once weekly are effective in the treatment of recalcitrant diabetic ulcers. **Key words:** *diabetic; fibroblast; keratinocyte; treatment; ulcer.*

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Numerous conservative and surgical methods have been developed for the treatment of chronic ulcers. Pinch grafting, split-thickness skin grafting and radical excision together with immediate skin grafting have been used successfully (1, 2). However, in addition to being a tissue replacement, the skin graft acts as a pharmacological agent stimulating wound closure with its growth factors. Thus, multiple autologous skin graftings may be needed for full closure of non-healing ulcers (3, 4).

Cultured allogeneic keratinocyte epithelium has been used for years for the treatment of burn injuries and chronic leg ulcers with different aetiologies (5–9). Allogeneic keratinocytes do not attach and cover the ulcer permanently and they are soon replaced by host keratinocytes. However, their role in

healing process is to accelerate epithelialization and granulation tissue formation (8). The possible stimulatory function of allogeneic fibroblasts is not as well documented, but they have been used in composite artificial skin preparations consisting of an extracellular matrix, allogeneic fibroblasts and autogeneic keratinocyte epithelium (10, 11).

The primary aim of this open study was to use cultured allogeneic keratinocyte epithelium for different diabetic leg and foot ulcers. Preliminary experiments were also performed with allogeneic fibroblasts grown in a gelatin matrix. Other chronic leg and ankle ulcers were treated in comparison with diabetic ulcers.

MATERIALS AND METHODS

Patients and source of allogeneic skin samples

The study included 21 leg and foot ulcer patients with either type I (8 patients) or type II (13 patients) diabetes under reasonable control (13 males and 8 females, mean age 58 ± 17 years). The mean duration of ulcers was 10.3 ± 15.8 (range 0.3–72) months and the size was 3.1 ± 6.6 cm² (range 0.1–34). In addition, a 71-year-old female with type II diabetes was recruited to the study, but was soon excluded due to erysipelas complication. The patients had been treated with conventional topical dressings with appropriate contact cast or orthoses if needed. Only patients without any other serious foot complications, such as deep infections, osteomyelitis or pregangraena, were included. Bacterial culture sample was obtained from each ulcer and, when necessary, antibiotic medication was used before initiation of the ulcer treatment. When necessary, a vascular surgeon was consulted. Fifteen patients out of 21 were not suitable for or did not need any operation for lower extremity arteries prior to the treatment. However, 5 patients of these 15 had undergone either percutaneous transluminal angioplasty or bypass surgery 6 months to 2 years earlier (Fig. 1). Four patients out of 21 had undergone percutaneous transluminal angioplasty 0.3–4 months earlier, but 2 of these 4 still had poor circulation. Two patients out of 21 were treated with angioplasty or bypass surgery during the ulcer treatment, but without any apparent effect on the rate of ulcer healing.

The second group consisted of 16 non-diabetic patients (3 males and 13 females, mean age 76 ± 8 years) with 16 chronic leg and 9 ankle ulcers. The mean ulcer duration was 6.8 ± 6.0 (range 1–24) months and the size was 10.5 ± 11.8 cm² (range 0.6–49). Five patients suffered from rheumatoid arthritis and were on anti-rheumatic medication. These rheumatic patients, however, did not show any clinical signs of active vasculitis. The aetiology of the ulcers of the remaining 11 patients was combined venous and arterial (2 patients), venous (3 patients), arterial (2 patients) and posttraumatic (4 patients). Because the aetiology of the chronic ulcers in previous studies (5, 6, 8, 9) has been widely variable, heterogeneous ulcers were also chosen for this study to compare the results.

The allogeneic skin samples were obtained as a left-over material from young boys in connection with a routine surgical operation on the foreskin. The parents of each child gave oral permission to use this skin material for the treatment of chronic ulcers after they had received written information. Each skin donor was tested for serum alanine aminotransferase levels, cardioplipin, hepatitis B surface anti-

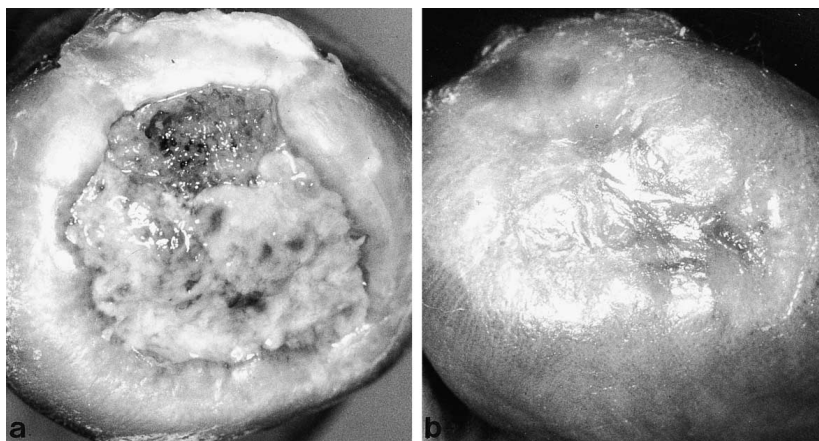


Fig. 1. A 54-year-old male with a history of type I diabetes for 31 years. Kidney transplantation was performed 8 years earlier, and the patient underwent percutaneous transluminal angioplasty for both legs 11 months earlier without an effect on wound healing. The patient repeatedly refused from amputation. (a) Chronic ulcer of 6-year duration on the tip of right big toe where the bone is exposed. (b) Healed ulcer after treatment with weekly keratinocyte epithelium 249 days later.

gen, hepatitis C antibodies, and HIV-1 and HIV-2 antibodies. In addition, a second blood sample was drawn 3 months later to test HIV antibodies again.

The protocol of this study was approved by the Ethics Committee of Kuopio University Hospital, Kuopio, Finland. The patients with chronic ulcers gave written informed consent.

Culture and preparation of skin cells

The allogeneic skin samples were stored for less than 5 days in a refrigerator in Keratinocyte-SFM[®] (Gibco BRL, Life Technologies Ltd, Paisley, UK) serum-free medium together with 100 µg/ml streptomycin, 100 U/ml penicillin and 2.5 µg/ml Fungizone[®] (Gibco). The tissue specimens were washed with Dulbecco's phosphate-buffered saline (D-PBS)(Gibco) followed by incubation in refrigerator with 2.4 U/ml dispase (Boehringer Mannheim GmbH, Mannheim, Germany) overnight. The epidermis was then easily separated from the dermis using forceps. The epidermis was further processed by incubating it in 0.05% trypsin and 0.02% EDTA (Viralex[®], PAA Laboratories GmbH, Linz, Austria) to release proliferating basal keratinocytes. After centrifugation, the cells were cultured in Keratinocyte-SFM[®] medium supplemented with 5 ng/ml epidermal growth factor, 50 µg/ml bovine pituitary extract, 100 µg/ml streptomycin and 100 U/ml penicillin, at 37°C and 5% CO₂, until subconfluence. Following detachment from plastic surface with 0.05% trypsin and 0.02% EDTA the cells were subcultured and expanded. Finally, aliquots of third to fourth passage keratinocytes were frozen in 15% dimethyl sulfoxide (Sigma, St Louis, MO, USA) and Medium 199 (Gibco) down to the temperature of liquid nitrogen.

Keratinocyte epithelium was prepared by culturing proliferating 3–8 passage keratinocytes in Petri dishes in Keratinocyte-SFM[®] medium with supplements. Upon confluence, the monolayer was induced to differentiate towards epithelium by culturing in 10% fetal calf serum (Viralex[®], PAA Laboratories GmbH), Dulbecco's-Modified Eagle Medium (DMEM) (Gibco), 100 µg/ml streptomycin and 100 U/ml penicillin for 1–2 weeks. Before clinical use, this medium was changed to DMEM alone 1 day earlier. Thereafter, the epithelium was washed with D-PBS, detached from plastic surface with 2.4 U/ml dispase for 15–20 min, washed again with D-PBS, and transferred to the treatment room.

The dermal part of the foreskin specimen was processed to fibroblast cultivation. The dermis was minced with scissors and then placed under a wire netting in a Petri dish. The outgrowth of fibroblasts from the dermis was followed by culturing in 10% fetal calf serum, DMEM, 100 µg/ml streptomycin and 100 U/ml penicillin for about 3–4 weeks.

Upon subconfluence, the cells were subcultured and expanded and finally stored in aliquots in liquid nitrogen.

The matrix for fibroblasts was prepared from pig skin gelatin (300 bloom, Sigma). One percent gelatin dissolved in deionized water was poured into glass Petri dish and then de-aerated. The gelatin gel was frozen and then vacuum dried. The prepared gelatin sponge was further dehydrothermally fixed in a vacuum oven at 110°C (12, 13). After overnight stabilization in CO₂ incubator, fibroblasts were seeded on the gelatin sponge by using 10% FCS, DMEM, penicillin and streptomycin as the growth medium for 2–3 weeks. Prior to clinical use, the medium was changed to DMEM with or without 50 µg/ml gentamycin (Gibco) 1–2 days earlier, and immediately preceding the transfer of the fibroblast-gelatin sponge to the patient's ulcer it was washed with DMEM.

Treatment of patients

The patient's ulcer was rinsed with warm tap water and saline and mechanically cleansed from debris at each visit. The size and shape of the ulcer was traced on a transparent film, its diameter and area were measured at each visit and it was photographed at regular intervals.

Tegapore[®] (3M Medical-Surgical Division, St Paul, MN, USA) nylon mesh was placed on the detached epithelium, which was then transferred onto the ulcer. The basal keratinocytes faced the ulcer surface. Wet saline dressing was placed on the Tegapore[®] mesh and covered with bandages. The patient was sent home immediately if capable, but was instructed to rest for the first 24 h if possible. No special restrictions were set on the daily home routine of the patient. If needed, a suitable shoe with orthoses was designed by a podiatrist to reduce weightbearing on the ulcer site. The bandages were removed by the patient or by a home nurse or by a nurse at a local healthcare centre after 3–5 days. The ulcer was inspected for possible side-effects and carefully cleansed, then covered with petrolatum gauze and dry bandages. This procedure was repeated once weekly or occasionally once every 2 weeks until the ulcer was healed or the treatment was judged non-effective. The treatment protocol with allogeneic fibroblasts cultured in gelatin sponge followed that with keratinocyte epithelium.

Statistical analysis

The Mann-Whitney U test was used to test the statistical significance between the groups. Linear association between the variables was tested with the Spearman correlation coefficients.

Table I. Comparison of the healing time of the diabetic ulcers treated with fibroblast-gelatin sponge alone, keratinocyte epithelium alone, or first with fibroblast-gelatin sponge followed by keratinocyte epithelium

	Fibroblast-keratinocyte group (n = 5)	Keratinocyte group (n = 18)	Fibroblast group (n = 3)
Number of treatments			
fibroblast	14 ± 4		5 ± 3
keratinocyte	15 ± 6	8 ± 7	
Healing time (days)			
50% closure	79 ± 35	22 ± 19	16 ± 11
100% closure	258 ± 140	64 ± 61	40 ± 22
Ratio (50% vs. 100% healing time)	0.342 ± 0.092	0.389 ± 0.183	0.383 ± 0.120

RESULTS

Treatment of diabetic ulcers

The purpose of this study was to heal the ulcer completely or to continue until no apparent healing could be observed despite repeated treatments. To obtain more detailed information about the healing process the time for 50% reduction in ulcer area was measured from the healing curve and a ratio of 50% vs. 100% healing time was calculated. A 71-year-old female with type II diabetes, with collapsed bone structure, probably due to an old trauma and neuroarthropathy, and subsequent neurotrophic sole ulcer with a size of 3.6 cm² developed an erysipelas infection. Therefore, the treatment was interrupted in a situation where the ulcer area was decreased by 33% within 50 days. This patient was excluded from further analysis of the results.

A total of 26 chronic ulcers in 21 patients were treated, and every diabetic ulcer (except 1 complication) healed during the study. Eighteen ulcers in 15 patients were treated with keratinocyte epithelium, and 3 ulcers in 2 randomly chosen patients with fibroblast-gelatin sponge alone. Five ulcers in 4 randomly chosen patients were treated with fibroblast-gelatin sponge first to the point where about 50% of the initial ulcer area was decreased (Table I). Due to the slow healing response the treatment was changed to keratinocyte epithelium to find out if the healing process could be improved. As seen in Table I, no difference in the ratio of 50% vs. 100% healing time was observed. Thus, the ulcers seem to heal with a certain rate regardless of the used cell type, and no further distinctions were made between keratinocytes and fibroblasts when assessing the results.

On an average, the patients were treated 12 ± 11 times once weekly. The time for 50% reduction in ulcer area varied from 5 to 112 days (32 ± 32 days, mean ± SD). However, the time for complete (100%) healing was about 3 times longer, and it varied from 14 to 386 days (99 ± 110 days). Figure 1 illustrates a representative case. The duration of ulcer correlated with the 100% healing time ($p = 0.001$, $r = 0.592$), but the ulcer size was not related in this regard ($p = 0.623$, $r = 0.101$).

To assess the association between healing rate and location of ulcer the patients were divided into 4 groups. The 50% and 100% healing times in the heel ulcer group ($n = 7$) were 67 ± 37 and 215 ± 140 days, respectively, which differ significantly ($p < 0.02$) from the corresponding values of 21 ± 7 and 41 ± 12 days in the Achilles+leg ulcer group ($n = 7$), 19 ± 27 and 76 ± 87 days in the toe ulcer group ($n = 7$) and 16 ± 10 and 47 ± 31 days in the sole ulcer group ($n = 5$). Thus, essentially

similar healing rates were observed regardless of whether the ulcer was in leg, toe or sole, but heel ulcers healed very slowly.

Treatment of non-diabetic ulcers

A total of 25 ulcers in 16 patients were treated with keratinocyte epithelium. In 2 ulcers only, fibroblast-gelatin sponge was used for the beginning of treatment. In these non-diabetic patients, the 50% and 100% healing times were 22 ± 17 and 77 ± 58 days (mean ± SD, $n = 20$, excluding non-healed ulcers), respectively, which were slightly shorter than those in diabetic patients (32 ± 32 and 99 ± 110 days). Furthermore, there were 4 subjects and 5 ulcers among these non-diabetic patients that did not heal completely despite prolonged treatment (for 165–246 days) although at least 60% ulcer closure was achieved in each case. A striking feature is that all these non-healed ulcers precipitated among patients who had been suffering from their ulcers for at least 1 year. The 50% healing time in the whole group ($n = 25$, including non-healed ulcers) was 26 ± 21 days, which is close to the 32 ± 32 days in diabetic patients. Although the duration of the ulcers did not differ significantly, the ulcer size in non-diabetic patients was significantly larger than that in diabetic patients ($p < 0.0001$). Also, the age of the patients was different ($p = 0.0017$).

DISCUSSION

Diabetic ulcers lead to markedly increased risk for lower extremity amputation (14). Thus, every effort should be made actively to stimulate the healing process. Several studies on growth factors in diabetic ulcer treatment have been published in recent years, but there are very few studies dealing with keratinocytes and fibroblasts in stimulating the healing. This study focused mainly on allogeneic keratinocyte epithelium, but preliminary experiments were also performed with fibroblasts. In addition, the present open study comprised diabetic ulcers of different aetiology, which reflects the patient material in clinical practice. A heterogeneous group of other chronic leg ulcers were also treated accordingly, although these 2 ulcer groups are not directly comparable.

Previous studies have shown the usefulness of allogeneic keratinocyte epithelium for the treatment of chronic leg ulcers with different aetiologies. Marcusson et al. (9) found in their study consisting of 42 patients with 52 chronic leg ulcers that 65% of these ulcers healed completely but the mean healing time varied from 5 to 32 weeks depending on the aetiological group. De Luca et al. (8) showed that 66.6% of the 30 ulcers in 21 leg ulcer patients healed within, on an average, 6.2 weeks. A

study by Teepe et al. (6) demonstrated that allogeneic keratinocyte epithelium could heal 83% of the 30 ulcers in 20 patients, with a mean healing time of 35 days. In addition, Phillips et al. (5) reported that, in 73% of the 36 ulcers in 23 leg ulcer patients with different aetiology, there was complete healing within 8 weeks but the mean healing time was 3.3 weeks. In this study by treating comparably heterogeneous ulcers, we found that 75% of the 25 ulcers in 16 non-diabetic patients healed completely with a mean healing time of 77 ± 58 days. Thus, the healing results of chronic leg ulcers in this study parallel those published previously. To summarize, allogeneic skin cells can heal 70–80% of the chronic leg ulcers within 1–3 months.

Diabetic foot ulcers present a difficult management problem due to their poor healing tendency. Often, these ulcers show no granulation tissue in the wound bed, and the first target is to stimulate angiogenesis and the extracellular matrix for subsequent epithelialization. Platelet-derived growth factor (PDGF) has been considered one of the most essential growth factors in wound healing. A double-blind, placebo-controlled multi-centre trial has recently shown that rh-PDGF-BB used daily in neurotrophic diabetic ulcers for a maximum of 20 weeks is effective; 48% of the ulcers healed in the study group and only 25% in the placebo group (15). The extracellular matrix-like preparation, Arg-Gly-Asp peptide matrix, used twice weekly in neurotrophic diabetic foot ulcers for a maximum of 10 weeks is also effective; it could heal 35% of the ulcers, whereas with placebo only 8% healed (16). However, living cells can carry numerous different growth factors and extracellular matrix components, which can be beneficial for wound closure. Therefore, attention has been paid to allogeneic skin cells in diabetic ulcers. The commercially available allogeneic cultured dermis (fibroblasts in polyglactin mesh, Dermagraft[®]) used weekly for full-thickness diabetic foot ulcers for 8 weeks has been shown to heal about 50% of the ulcers within 12 weeks (17, 18). The healing results in this study are comparably close and 58% (11/19) of the diabetic foot ulcers healed by 12 weeks. However, the study groups can not be compared directly; the patients in the previous studies (17, 18) did not have vasculopathy but had neuropathic ulcers. It is not known whether keratinocytes could be superior to fibroblasts or vice versa. Also, a composite artificial skin consisting of keratinocytes, fibroblasts and extracellular matrix skeleton may be even more optimal than use of these cells alone or in sequential order. We performed preliminary experiments to address this question (Table I) but could not find any clear difference between these cells. Therefore, both allogeneic keratinocytes and fibroblasts are useful to force closure of diabetic ulcers. However, the optimal metabolic activity of the cells in the artificial skin preparation seems to determine the success of wound closure (18).

Both 50% and 100% healing times for diabetic ulcers were slightly longer than those for non-diabetic ulcers. This may suggest that diabetes does not interfere markedly with the wound healing during treatment. However, the ulcers were larger and the non-diabetic patients were older than the diabetic patients. Thus, with smaller ulcer size and lower age these non-diabetic ulcers might heal more rapidly. Nevertheless, all non-diabetic ulcers were located in or above ankle level, but 73% of the diabetic ulcers in heel, sole or toe, which are usually very treatment-resistant areas. Furthermore, 5 non-diabetic ulcers of 25 could not be healed completely. When these facts are summarized it could be concluded that the healing of diabetic

ulcers with cultured skin cells is comparable to that of other chronic leg ulcers.

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