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Allogeneic Cultured Keratinocytes in the Treatment of Leg Ulcers

A Pilot Study

MARCUSSON JAN A[2], LINDGREN CARIN[1], BERGHARD ANNA[1] and TOFTGÅRD RUNE[1]

[1]Center for BioTechnology, Karolinska Institute, NOVUM and [2]Department of Dermatology, Huddinge Hospital, Huddinge, Sweden

Forty-two patients (10 males and 32 females) with 52 chronic leg ulcers were treated with sheets of cultured allogeneic keratinocytes. Sixty-five % of the ulcers healed completely and the healing rate differed between various diagnostic groups. The best results were obtained in patients with venous ulcers and wounds with mixed etiology, whereas less improvement was observed with ischaemic ulcers. Rheumatic ulcers also responded well in combination with oral corticosteroids. The overall impression was that the grafting procedure markedly enhanced wound healing. ***Key words: Allograft; Wound healing.***

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J. A. Marcusson, Department of Dermatology, Huddinge Hospital, S–141 86 Huddinge, Sweden.

Recent Swedish studies have shown that the prevalence of leg and foot ulcers in the Gothenburg area was between 0.2 and 0.4% (1–3). Similar figures have been obtained in another investigation (4). The frequency of ulcer occurrence increases with patient age and it has been shown that the number of ulcers with arterial etiology has increased over the last 10 year period (2, 3). The cost of care of ulcer patients is high and will increase as the elderly population increases (5), thus new methods with the potential to improve the healing process are greatly needed. Topically applied autologous and allogeneic cultured keratinocytes have been used for healing wounds such as burns, leg ulcers and wounds after surgical excision of skin (6). Autologous cultured keratinocytes seem to act like a transplant and the graft has been shown to "take" (6). *In situ* hybridisation studies using a Y-chromosome specific probe and patients and donors of opposite sex have demonstrated that this is not the case with allogeneic grafts (7, 8). Cultured allogeneic keratinocyte sheets have been reported to enhance healing of leg ulcers (9-13). We have evaluated this method on unselected patients with leg ulcers of varying etiology.

MATERIALS AND METHODS

The patient material consisted of 42 subjects (10 males and 32 females). The median age was 71 years (range 46–89). All subjects were out-patients at the leg ulcer clinic of the Dermato Venereology Clinic at Huddinge Hospital and belonged to various diagnostic groups. The ulcers were situated on the lower legs and feet and the patients were categorized as follows (for details see Table I):

Venous ulcers

Ulcers situated at the medial distal third of the legs; ankle pressure (AP) > 100 mmHg, ankle index (AI; systolic blood pressure of the ankle/systolic blood pressure of the arm) > 0.9.

Ischaemic ulcers

AP mostly < 100 mmHg and AI < 0.9.

Combined ulcers

Showing features of a venous ulcer but AP < 100 mmHg and AI < 0.9.

Foot ulcers

Ulcers situated on the feet below the shoe line and malleoli.

Mixed etiology ulcers

Wounds which did not fit into the above categories and which had an apparent traumatic or postinfectious cause. One patient had necrobiosis lipoidica and two ulcers in a transplanted area.

Rheumatic wounds

Long-standing wounds and concomitant rheumatic arhritis. AP > 100 mmHg and AI > 0.9.

Keratinocyte culture

The skin donors were neonatal and the mothers had been tested for HIV and Hepatitis B. Keratinocytes were prepared from human foreskin by enzymatically dissociating epidermis from dermis with 0.5% Pronase (Boehringer), at 37°C for 20–40 min. Cells were seeded on lethally irradiated Swiss 3T3 mouse fibroblasts as described by Green *et al.* (14) and grown in 5% CO_2 at 37°C. The medium consisted of Dulbecco's modified Eagles medium (DMEM): Hams F12 (3:1; Gibco or Nordvacc) supplemented with 5% fetal calf serum (Gibco), 5 µg/ml insulin (Sigma), 0.4 µg/ml hydrocortisone (Sigma), 10 ng/ml epidermal growth factor (Sigma), 10^{-10} M cholera toxin (Sigma), and 1.8×10^{-4} M adenine (Sigma). The medium for primary cultures also contained penicillin/streptomycin (100 IU/100 µg × ml^{-1}; Nordvacc) and Fungizone (0.25 µg × ml^{-1}; Gibco). The keratinocytes were split 1:4 before reaching confluency and no feeder cells were used in the second or third passages. Before the keratinocyte sheets were used for grafting, cholera toxin was excluded from the media.

The keratinocyte sheets used for grafting consisted of 1 basal and 1–2 suprabasal cell layers and were prepared from cells in the second or third passage. The cells were detached from the plastic in intact sheets by using Dispase II (0.4%, 5 min, room temperature; Boehringer), rinsed in sterile phosphate buffered saline and "backed" on vaseline gauze.

Patient care and grafting procedure

The wounds were prepared for grafting in the following way: for debridement, a CO_2 laser was mostly used with superficial evaporisation (1–4 Watt, continuous ray); sometimes curettage was done after wound occlusion with hydrocolloid dressings. No grafting was performed until at least one week after these procedures. Cellutitis was treated with appropriate antibiotics. In the case of leg swelling and thick wound edges, active therapy with sequential pneumatic compression was given for 30–90 min (Turbo Puls System AB, Uppsala, Sweden). During the treatment the wounds were covered with gauze soaked in chlorhexidine solution (0.25%). The usual pressure was 70–80 mmHg. Both the pressure and time was adjusted to avoid pain in the case of ischaemia. Prior to grafting, the area of the wound was traced using transparent film and then measured using Image 1.22 (public domain developed by W. Rasband, NIH, research services Branch), and a Mac II with a flatbed scanner (Microtek).

Table I. *The patient's material and results. The values shown are median values and the ranges are given within parenthesis*

No of wounds (healed/non healed)	Ankle pressure (mmHg)	Ankle index	Wound duration before grafting (weeks)	Wound area (cm^2)	Healing time (weeks)	Number of keratinocyte graftings
Venous ulcers (M=2, F=11; Age: 70 (49–89))						
17	175	1.20	50	5.4	9	5
(13/4)	(110–300)	(0.90–1.67)	(15–156)	(0.7–34.1)	(5–26)	(1–10)
Ischaemic ulcers (M=1, F=5; Age: 81 (80–86))						
7	100	0.65	156	12.6	32	6
(1/6)	(90–150)	(0.54–0.80)	(26–156)	(3.5–55.1)		(1–15)
Combined ulcers (M=1, F=1; Age: 64 (62–74))						
2	75	0.6	34	8.1	21	3
(2/0)	(70–80)	(0.5–0.7)	(16–54)	(0.7–15.5)	(3–40)	(1–5)
Foot ulcers (F=5; Age: 82 (63–85))						
8	210	1.40	16	8.8	7	3
(3/5)	(110–260)	(0.80–1.60)	(12–26)	(0.6–17.1)	(6–8)	(2–5)
Mixed etiology (M=3, F=7; Age: 73 (43–80))						
10	160	1	23	9.3	5	3
(8/2)	(120–200)	(0.67–1.14)	(12–156)	(1.1–188)	(3–28)	(1–11)
Rheumatic wounds (M=3, F=3; Age: 64 (48–83))						
8	165	1.07	20	8.5	24	7
(7/1)	(120–220)	(0.75–2.56)	(10–156)	(2.1–112)	(4–40)	(1–10)

M and F indicate number of male and female patients

The sheets of keratinocytes were applied directly on the wound bed with the basal cells down. The sheets were overlaid with gauze pads to establish close contact between the graft and the wound bed. A firm elastic bandage was applied to keep the graft in place. Patients were asked to remain in bed for 3–4 days and then the wound was inspected. If there was no secondary infection or eczema of the surrounding skin, the ulcer was soaked in saline without removal of the graft. The dressing was changed every second day. If complications occurred the dressing was changed daily. When the ulcers were covered with new epithelium they were defined as healed. The study was performed during September 1989 to May 1990.

RESULTS

Thirty-four out of 52 ulcers (65%) were completely healed (Table I and Fig. 1). The healing rate differed within the various categories. The most promising results were obtained in the venous ulcer group which showed 50% healing within 3 months compared with 40% healing of all wounds (Fig. 1).

Venous ulcers

Thirteen of 17 ulcers healed completely in 13 patients and the median healing time was 9 weeks. The median wound area of the healed ulcers was 3.1 cm^2 (range 0.7–5.8) and of the non-healed ulcers 12.3 cm^2 (range 2.8–34.1). Among the four wounds that did not heal, 2 increased their areas by 32% and 110%, respectively; one decreased by 50% and one ulcer remained unchanged. The reason for the incomplete healing could be due to concomitant diseases such as secondary atrophy blanche, swelling of the lower legs due to heart incompensation and swelling due to lymphoedema. The larger starding wound area in the latter case could also negatively influence the results.

Ischaemic ulcers

Out of 7 ulcers 1 healed after 32 weeks; 10 graftings were performed. Among the other 6, three increased in size (7–55%) and 3 decreased (34–60%). The AP and AI in the healed subject was 90 mmHg and 0.8 respectively; the median AP and AI in the non-healing group was 110 mmHg (90–150) and 0.65 (0.54–0.8). The initial areas of the ulcers were 3.8 cm^2 in the healed group and 16 cm^2 (3.5–55) in the non-healed group.

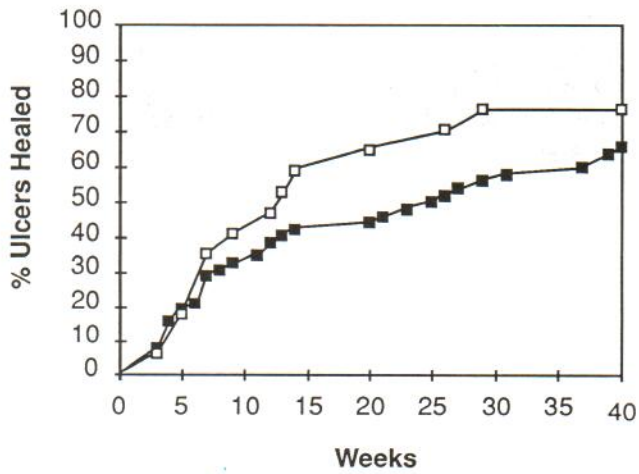

Fig. 1. Illustrates the rate of healing in all ulcers (■) and venous ulcers (□); note that 50% of the venous ulcers were healed within a 3-month period.

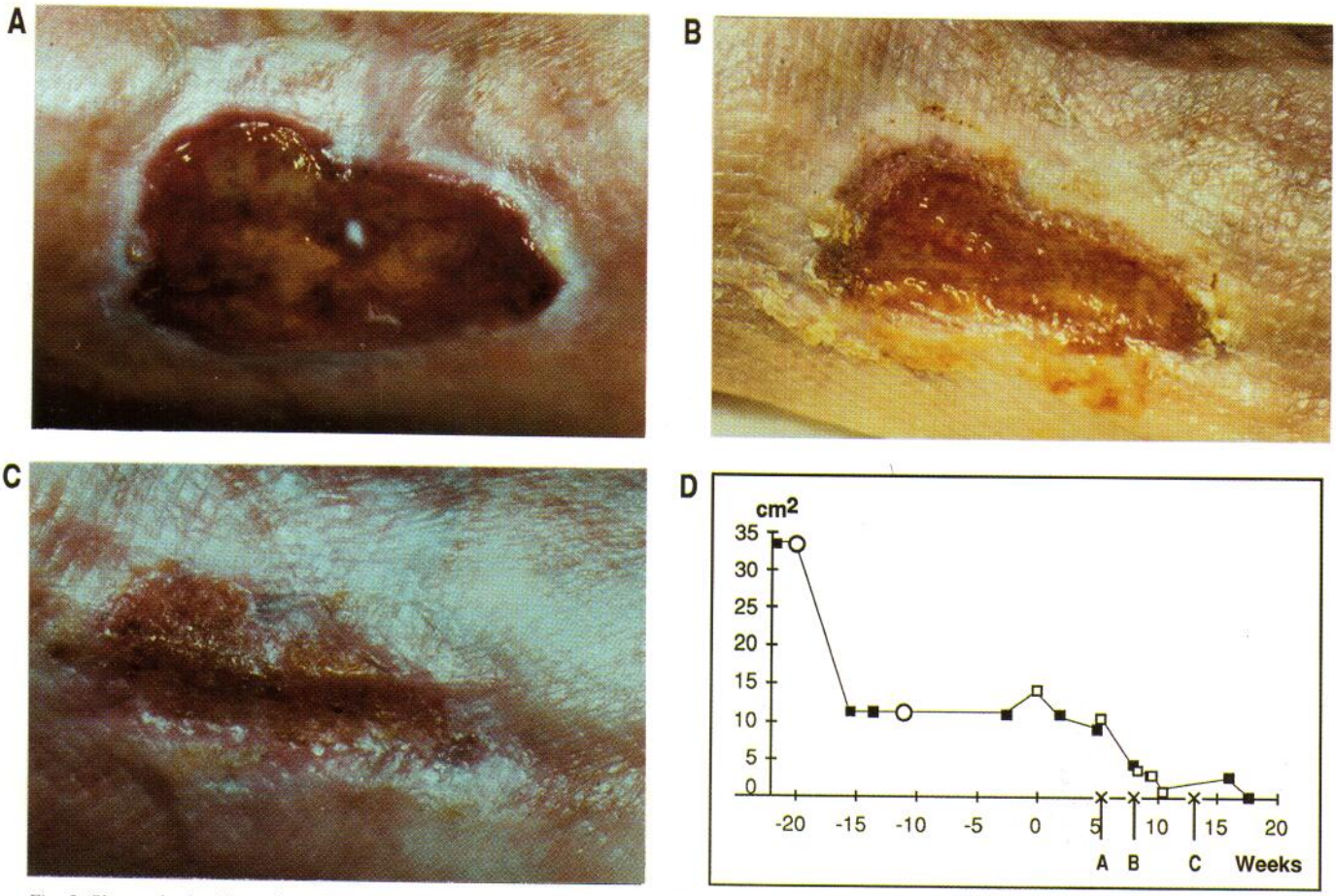

Fig. 2. Shows the healing of an ulcer with mixed etiology. The duration of this ulcer was 20 months and it was previously treated by conventional methods including pinch grafting. A) Five weeks after receiving 1 keratinocyte graft. B) Eight weeks after graft treatment started. The patient was given 2 grafts. C) Thirteen weeks and 5 grafts. D) The chart shows the healing rate of the ulcer shown in panels A–C. (□) denotes that a keratinocyte graft has been received, (■) ulcer area measurement; (○) indicates that a pinch graft was received. A, B, C on the X-axis mark the time points of when the photos were taken.

Combined ulcers

All ulcers in this group healed completely (Table I).

Foot ulcers

Three out of 8 wounds healed. The median wound area in the healed group was 1.9 cm^2 (0.9–5.8) and in the non-healed group 11.9 cm^2 (4.2–17.1). Two of the patients in the non-healing group had wounds which were of the secondary atrophy blanche type.

Mixed etiology ulcers

Eight out of 10 ulcers in this group healed after treatment and in the remaining 2 there was a reduction of the wound area by 60%. One patient that healed had an AI of 0.67 but the AP was 120 mmHg.

Rheumatic wounds

Seven out of 8 wounds in this group healed completely; one increased in size by 60%. Concomitant therapy was given with prednisolone (15–25 mg/day) or cyclophosphamide (100 mg/day). Of two patients treated with plasmapheresis, one healed completely and the other patient's ulcer increased in size.

Case report

A 77–year-old female with a longstanding (20 months) ulcer with mixed etiology (AI 1.0; AP 170 mmHg) had been given conventional treatment with no promoting effect on healing. Pinch grafting initially gave a reduction of ulcer area from 35 cm^2 to 10 cm^2. During the following 15 weeks, the healing process stopped but restarted when keratinocyte grafts were applied (Fig. 2).

DISCUSSION

Our results indicate that the keratinocyte grafts were most effective in stimulating healing of venous ulcers (13 to 17), ulcers with mixed etiology (8 to 10) and rheumatic ulcers (7 of 8). Less improvement was demonstrated for ischaemic ulcers. Large ulcers seemed to respond less well compared to smaller ones but in most cases a significant reduction in size was observed. The data indicate a marked positive effect which is in agreement with previous studies (9–13). An interesting observation is the complete healing in the combined ulcer group, despite the low AP and AI (Table I). This indicates that it is not possible to exclude those patients from treatment with cultured allograft since the end results are not obvious until

the grafting procedure has been tried. A combined treatment was used in the venous and rheumatic ulcer groups. Thus patients with venous ulcers received active compression therapy which might have contributed to the healing. Adjuvant therapy was also given to patients with rheumatic ulcers; in addition to keratinocyte grafting they have been treated with either prednisolone, cyclophosphamide or plasmapheresis.

The data clearly show that the selection of patients is important since the result vary between the different diagnostic groups. This puts into focus the need to evaluate the beneficial clinical effect of keratinocyte grafting compared to other treatments in a controlled study with well defined clinical patient groups.

The clinical impression is that grafting with allogeneic cultured keratinocytes accelerates the healing process. We confirm the previously reported observations concerning "edge" and "central" wound effects (10–12). The "edge" effect (Fig. 2C) was obvious and manifested itself as a translucent blue/red edge at the periphery of the wound consisting of epithelium growing towards the wound center. The "central" effect (Fig. 2A) presented itself as islands of epithelium in the central part of the ulcer from which the epithelium grew out radially. The latter phenomenon is believed to represent keratinocyte outgrowth from the hair follicles.

No obvious clinical "take" and no signs of graft rejection have been registered. However, we cannot exclude that an immunological reaction has occurred and contributed to the effect of the graft, for example through the release of cytokines. A recent study (16) using an *in vitro* dermal explant model for studying stimulatory effects of mitogens and cultured allogeneic keratinocyte sheets on re-epithelisation showed that the stimulatory effect of the cultured cells was greater than that of the mitogens. Since the cultured keratinocyte allografts do not remain permanently on the wound it must be assumed that they in some way stimulate the proliferation and/or migration of the host keratinocytes. This stimulation could be due to factors secreted by the cultured keratinocytes, cell-cell or cell-matrix interactions.

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