

Autologous full-thickness skin substitute for healing chronic wounds

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Summary

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Conflicts of interest

None declared.

Background Chronic wounds represent a major problem to our society. Therefore, advanced wound-healing strategies for the treatment of these wounds are expanding into the field of tissue engineering.

Objectives To develop a novel tissue-engineered, autologous, full-thickness skin substitute of entirely human origin and to determine its ability to heal chronic wounds.

Methods Skin substitutes (fully differentiated epidermis on fibroblast-populated human dermis) were constructed from 3-mm punch biopsies isolated from patients to be treated. Acellular allodermis was used as a dermal matrix. After a prior 5-day vacuum-assisted closure therapy to prepare the wound bed, skin substitutes were applied in a simple one-step surgical procedure to 19 long-standing recalcitrant leg ulcers (14 patients; ulcer duration 0.5–50 years).

Results The success rate in culturing biopsies was 97%. The skin substitute visibly resembled an autograft. Eleven of the 19 ulcers (size 1–10 cm²) healed within 8 weeks after a single application of the skin substitute. The other eight larger (60–150 cm²) and/or complicated ulcers healed completely (n = 5) or continued to decrease substantially in size (n = 3) after the 8-week follow-up period. Wound healing occurred by direct take of the skin substitute (n = 12) and/or stimulation of granulation tissue/epithelialization (n = 7). Skin substitutes were very well tolerated and pain relief was immediate after application.

Conclusions Application of this novel skin substitute provides a promising new therapy for healing chronic wounds resistant to conventional therapies.

Chronic wounds, including venous and arterio(lo)sclerotic leg ulcers, diabetic foot ulcers, decubitus and trauma-induced wounds, represent a major burden in our society. These wounds occur with high incidence and exist for prolonged periods of time and therefore have a great socioeconomic impact. One to two per cent of the general population and more than 5% of those over 80 years of age are affected. The problem increases as the average age of the population increases and therefore new therapies in wound healing are continuously being sought.¹

Advanced methods in wound care include biomaterial dressings and skin substitutes.^{2–6} The advantage of a living skin substitute above an acellular dressing is now widely accepted: living skin substitutes secrete a cocktail of cytokines, chemokines and growth factors which promote wound healing (by stimulating angiogenesis, granulation tissue formation and epithelialization) as well as providing an immediate cover

for the wound. There are currently three categories of living skin substitute available: allogeneic or autologous fibroblast-populated dermal substitutes,^{7,8} allogeneic or autologous epidermal substitutes^{9–11} and allogeneic full skin substitutes (allogeneic epidermis on allogeneic fibroblast-populated bovine collagen gels).¹² No autologous, full-thickness skin substitute of entirely human origin and which closely resembles a native autograft (frequently used in acute wound healing) is available. The advantage of such a skin substitute above a native autograft is that less donor skin is required and that the wound-healing properties of the skin substitute can be modified for the type of wound to be treated: for example, chronic wounds where healing of the inert ulcer is of most importance require a different skin substitute compared with burn wounds where scar formation has to be taken into consideration. The aim of this study was to develop an autologous, full-thickness skin substitute and to evaluate its efficiency and

applicability in closing long-standing ulcers that had proven nonresponsive to the currently available wound-healing therapies.

Materials and methods

Construction of autologous skin substitute

Epidermal sheets and dermal fibroblasts were used to construct autologous skin substitutes. For each 1.5 cm² of autologous skin substitute to be constructed, a single 3-mm biopsy obtained from the patient's upper leg was required. These donor biopsy sites healed without complications, and sutures were not required. For patients 4, 6, 13 and 14 a larger skin sample was removed from the abdominal region and was subsequently punched *ex vivo* into numerous 3-mm biopsies (Table 1). In order to obtain additional data on the efficiency of skin substitute construction, neonatal foreskins and skin

from leg, back, breast, face or ear were obtained from routine surgical procedures (from nonincluded patients) and further treated in an identical manner to the material from included patients (Table 1). Acellular human allodermis was prepared from glycerol-preserved donor skin (Euro Skin Bank, Beverwijk, the Netherlands).

Epidermal sheets and dermal fibroblasts were separated by incubation in dispase II (Roche, Mannheim, Germany) overnight at 4 °C as previously described.¹³ Skin substitutes were constructed as described in Patent International Publication No. WO 2005/068614 A2. In short: epidermal sheets were placed stratum corneum side upwards on the allodermis and cultured, exposed to the air, in keratinocyte medium consisting of Dulbecco's modified Eagle's medium (DMEM) (ICN Biomedicals, Irvine, CA, U.S.A.): Ham's F12 (ICN Biomedicals) (3 : 1) containing 1% UltroSerG (BioSeptra SA, Cergy-Saint-Christophe, France), 1 µmol L⁻¹ hydrocortisone, 1 µmol L⁻¹ isoproterenol, 0.1 µmol L⁻¹ insulin, 4 ng mL⁻¹ keratinocyte

Table 1 Efficiency of culture

Patient/donor	Sex	Age (years)	No. of biopsies taken	No. of biopsies cultured successfully
1 ^a	M	72	2	2
2 ^a	F	85	9	7
3 ^a	F	70	4	4
4 ^b	F	85	100 (10 cm ²)	100
5 ^a	F	73	4	4
6 ^b	F	78	60 (6 cm ²)	60
7 ^a	F	79	6	5
8 ^a	M	50	9	9
9 ^a	F	79	4	4
10 ^a	M	55	3	3
11 ^a	M	39	4	4
12 ^a	F	64	3	3
13 ^b	M	54	40 (4 cm ²)	40
14 ^b	F	77	50 (5 cm ²)	50
15 ^c	F	72	4	4
16 ^b	M	72	2	2
17 ^d	M	73	4	4
18 ^c	F	53	12	12
19 ^f	M	82	4	4
20 ^a	F	47	10	10
21 ^a	M	53	10	10
22 ^f	F	78	16	16
23 ^b	M	61	32 (4 cm ²)	32
24 ^b	M	54	4	4
25 ^a	M	67	4	4
26 ^a	F	54	8	5
27 ^g	M	<4	8	7
28 ^g	M	<4	8	8
29 ^g	M	<4	8	8
30 ^g	M	<4	8	8
31 ^g	M	<4	8	8

1–14: Skin obtained from included patients (see Table 2); 15–31: skin obtained from nonincluded patients during routine surgical procedures. This skin illustrates the flexibility of potential donor sites: it was biopsied (3 mm) in the laboratory and further handled as skin from included patients. Skin donor sites were: ^aupper leg; ^babdomen; ^cear; ^dface; ^eneck; ^fback; and ^gforeskin. All skin samples were received in the culture laboratory 2–48 h after the surgical procedure.

growth factor, 1 ng mL⁻¹ epidermal growth factor, 50 IU mL⁻¹ penicillin G (Gibco, Paisley, U.K.) and 50 µg mL⁻¹ streptomycin (Gibco) as previously described.¹⁴ Primary fibroblasts were cultured in 0.4-µm pore size transwells (Cat. no. 3450; Costar Corning Incorporated, Acton, MA, U.S.A.) until 70% confluent (approximately 7 days) in DMEM containing 1% UltraSerG, 50 IU mL⁻¹ penicillin G and 50 µg mL⁻¹ streptomycin. After approximately 7 days of culturing the primary fibroblast culture and epidermal sheet apart, the allodermis containing the epidermal sheet was placed on to the fibroblasts in order to allow fibroblast migration into the reticular side of the dermis. The skin substitute was further cultured in DMEM: Ham's F12 (3 : 1) containing 0.2% UltraSerG, 1 µmol L⁻¹ hydrocortisone, 1 µmol L⁻¹ isoproterenol, 0.1 µmol L⁻¹ insulin, 1.0 × 10⁻⁵ mol L⁻¹ L-carnitine, 1.0 × 10⁻² mol L⁻¹ L-serine, 1 µmol L⁻¹ DL-α-tocopherol acetate, 50 IU mL⁻¹ penicillin G, 50 µg mL⁻¹ streptomycin and enriched with a lipid supplement containing 25 µmol L⁻¹ palmitic acid, 15 µmol L⁻¹ linoleic acid, 7 µmol L⁻¹ arachidonic acid and 24 µmol L⁻¹ bovine serum albumin. Keratinocyte growth factor (4 ng mL⁻¹) and epidermal growth factor (1 ng mL⁻¹) were added to the culture medium for the next 7 days. Penicillin/streptomycin was omitted 4 days before application to the wound. Culture medium was renewed twice weekly. Unless otherwise stated, all culture additives were obtained from Sigma (St Louis, MO, U.S.A.). Skin substitutes were ready for application 3 weeks after removal of the skin biopsies from the patients. During this 3-week culture period the epidermis had expanded to cover the allodermis, resulting in approximately 20-fold amplification of the original surface area of the epidermal sheet (1 × 3 mm biopsy produced 1.5 cm² skin substitute). A quality control involved (immuno-) histological analysis of a 3-mm punch biopsy obtained from the cultured skin substitute before application (see below).

Immunohistochemical staining and determination of proliferation index

Immunohistochemical staining of paraffin sections using antibodies directed against Ki67 (Dako, Glostrup, Denmark) and AS02 (Dianova, Hamburg, Germany), and freeze sections using antibodies directed against α-smooth muscle actin (α-SMA) (Sigma-Aldrich, Zwijndrecht, the Netherlands), heparan sulphate proteoglycan (HSPG) (Abcam Ltd, Cambridge, U.K.), collagen IV (Monosan, Uden, the Netherlands), collagen VII (Novocastra, Newcastle upon Tyne, U.K.) and BP180 antigen (collagen XVII) (generous gift from Dr K. Owaribe, Japan) was performed as previously described.^{15,16} The proliferation index was established as the ratio of Ki67+ nuclei to the total number of basal cells (×100%), as described previously.¹⁶

Clinical application

We included 14 patients with long-standing ulcers, nonresponsive to short stretch (adhesive) bandages (in the case of venous leg ulcers) and to wound dressings, e.g. hydrocolloids,

alginates, antibiotics and antiseptics, who had been attending our outpatient department weekly for at least 8 weeks without any tendency of healing. Excluded were patients with penicillin allergy, general disease affecting the short-term prognosis, and patients refusing in-hospital treatment and/or frequent visits. Usually patients had already undergone venous surgery and sometimes application of grafts (split-thickness or punch grafts). For patient aetiology see Table 2. In total, 19 ulcers from 14 different patients were included for treatment with autologous skin substitutes (Tables 1 and 2). All patients received vacuum-assisted closure (VAC) therapy for 5 days prior to application of the skin substitute in order to clean the wound and stimulate granulation tissue formation. The skin substitute was applied to the wound and held in place with Lomatuell® H (Lohmann and Rauscher, Rengsdorf, Germany) and povidone-iodine ointment (Betadine®; Mundipharma AG, Basel, Switzerland). A short stretch bandage was applied and bed rest recommended for 5 days. Standard compression therapy with short stretch (adhesive) bandages was continued thereafter. Time to 100% healing was defined by full re-epithelialization of the wound and no drainage from the site. All procedures were performed with the Ethics Committee approval and patient consent.

Results

Characteristics of the autologous tissue-engineered skin substitute

The aim of this study was to develop an autologous full-thickness skin substitute that visibly resembles human skin and to test its efficacy in chronic wound healing. Because patient material and logistics are limiting factors for clinical procedures, both fibroblasts and keratinocytes are isolated from a single biopsy. Acellular donor dermis is used as a dermal matrix for reconstructing the skin. Autologous skin substitutes are constructed over a period of 3 weeks. During this time the expanding epidermis, originating from the intact epidermal sheet, covers the dermis. A cross-section shows that the epidermis is fully differentiated and visibly represents healthy human skin (Fig. 1a). It consists of a compact basal layer, spinous layer, granular layer and stratum corneum. Proliferating keratinocytes are located in the basal layer (see Ki67 immunostaining) and the proliferation index of the epidermis (9.9 ± 1.0% of basal keratinocytes; n = 6) corresponds closely to that found in healthy skin (11.3 ± 2.1%; n = 6). Fibroblasts populate the allodermis with a similar distribution to that observed in healthy skin (see AS02 immunostaining). α-SMA is not expressed by the fibroblasts, indicating that no differentiation to myofibroblasts has occurred that could facilitate hypertrophic scar formation in the patient to be treated. Figure 1a also shows α-SMA expression in smooth muscle cells lining blood vessels of human skin: the staining of these cells is absent in the skin substitute, indicating that smooth muscle cells are not present in the skin substitute.

Table 2 Efficiency of wound healing after application of skin substitute^a

Patient	Ulcer type	Previous vascular surgery and grafts ^a	Duration (years)	Ulcer area (cm ²)	Healing after 8 weeks (%)	Complete healing (weeks)
1	Venous, diabetes mellitus	None	2	3	100	6 ^d
2	Venous, diabetes mellitus	Punch grafts	1.5	10	70	9 ^e
3	Venous/arteriosclerotic	Multiple venous surgery, punch grafts	30 ^b	5	100	7 ^d
4	Venous/arteriosclerotic	Multiple venous surgery, dotter	50	150	40	32 (40%) ^{e,f}
			50	3	100	2 ^d
			50	3	100	4 ^d
5	Venous	Venous surgery, punch grafts	30 ^b	6	100	3 ^d
6	Post-thrombotic, protein C deficiency	None	15	130	85	12 (95%) ^{e,f,g}
7	Post-thrombotic/arteriosclerotic	None	20	7	80	16 ^e
8	Post-thrombotic	Venous surgery; split skin autograft; punch grafts	1	10	100	8 ^d
			1	1	100	4 ^d
9	Post-thrombotic	None	0.5	1	100	3 ^d
			15 ^b	2	100	4 ^e
			15 ^b	1	85 (4 weeks) ^c	4 ^{c,e}
10	Decubitus heel	None	1	3	100	2 ^d
11	Decubitus (after leg amputation)	None	0.5	5	100	6 ^d
12	Persistent wound after major surgery (trauma)	Autograft	5	3	75	12 (80%) ^d
13	Persistent wound after orthopaedic surgery, diabetes mellitus	2× split-skin autograft, punch grafts	2.5	60	50	17 ^e
14	Hydroxyurea use (polycythaemia vera)	None	0.5	100	60	24 ^d

^aAll patients had compression therapy and were treated with diverse local ointments/wound dressings; ^brecurrent ulcer; ^cafter 4 weeks patient stopped attending the day care clinic as ulcers were healed or almost healed; ^dskin substitute completely incorporated into wound bed; ^eskin substitute rejected in part or totally at 2–6 weeks after application; ^fulcer showed partial healing; ^gafter 12 weeks ulcer was 95% healed, and ulcer was completely healed after 11 months.

Analysis of the dermal-epidermal junction of the skin substitute shows that basement membrane proteins HSPG and collagens IV and VII are expressed in the allodermis and remain intact during construction of the skin substitute (Fig. 1b). The hemidesmosomal protein BP180 (collagen XVII) is synthesized and deposited *in vitro* by keratinocytes in a similar manner to healthy skin. The correct deposition of these proteins is essential for epidermal growth and cell migration and also for the skin substitute to remain intact upon application without blistering.

Application of the autologous skin substitute

Having developed the skin substitute we next determined the efficiency of the culture method and its capacity to heal inert ulcers. Skin substitutes were successfully constructed from biopsies obtained from patients/donors aged up to 85 years. Table 1 shows that the success rate in culturing the biopsies was 97%.

Skin substitutes were applied to 19 lower leg ulcers (14 patients) (Table 2; Fig. 2). All patients reported a substantial and immediate pain reduction. Within 1 week after application the epidermis of the skin substitutes expanded and migrated on to the patient's surrounding skin (Fig. 2a). In 12 of 19 ulcers the skin substitutes were not rejected but were com-

pletely incorporated into the wound bed. The median time for healing was 6 weeks, and healing resulted in visibly normal-appearing skin in the previously ulcerated area. Of these 12 ulcers only one failed to heal completely within 24 weeks (patient 12). In the other seven ulcers, 2–6 weeks after application the skin substitutes could be partially or entirely removed from the wound bed and therefore they were not incorporated. Wound healing had been stimulated to such a degree that four of these seven ulcers healed (median time for healing was 16 weeks) and the other three ulcers decreased significantly in size (see Table 2). These included two larger and more complicated ulcers that are currently still healing. One of these ulcers did continue to heal completely and was completely closed 11 months after application of the skin substitute. The other showed good granulation tissue and re-epithelialization from the wound margins. No correlation could be found between duration of ulcer or type of ulcer and the time taken for complete healing. However, in general, larger ulcers did take longer to heal than smaller ulcers. After healing, all patients with venous problems were treated with therapeutic elastic stockings (compression class 2 or 3). In a follow-up period of 6 months one patient (patient 11) had a recurrence of his ulcer, most probably due to continuous friction from wearing a leg prothesis.

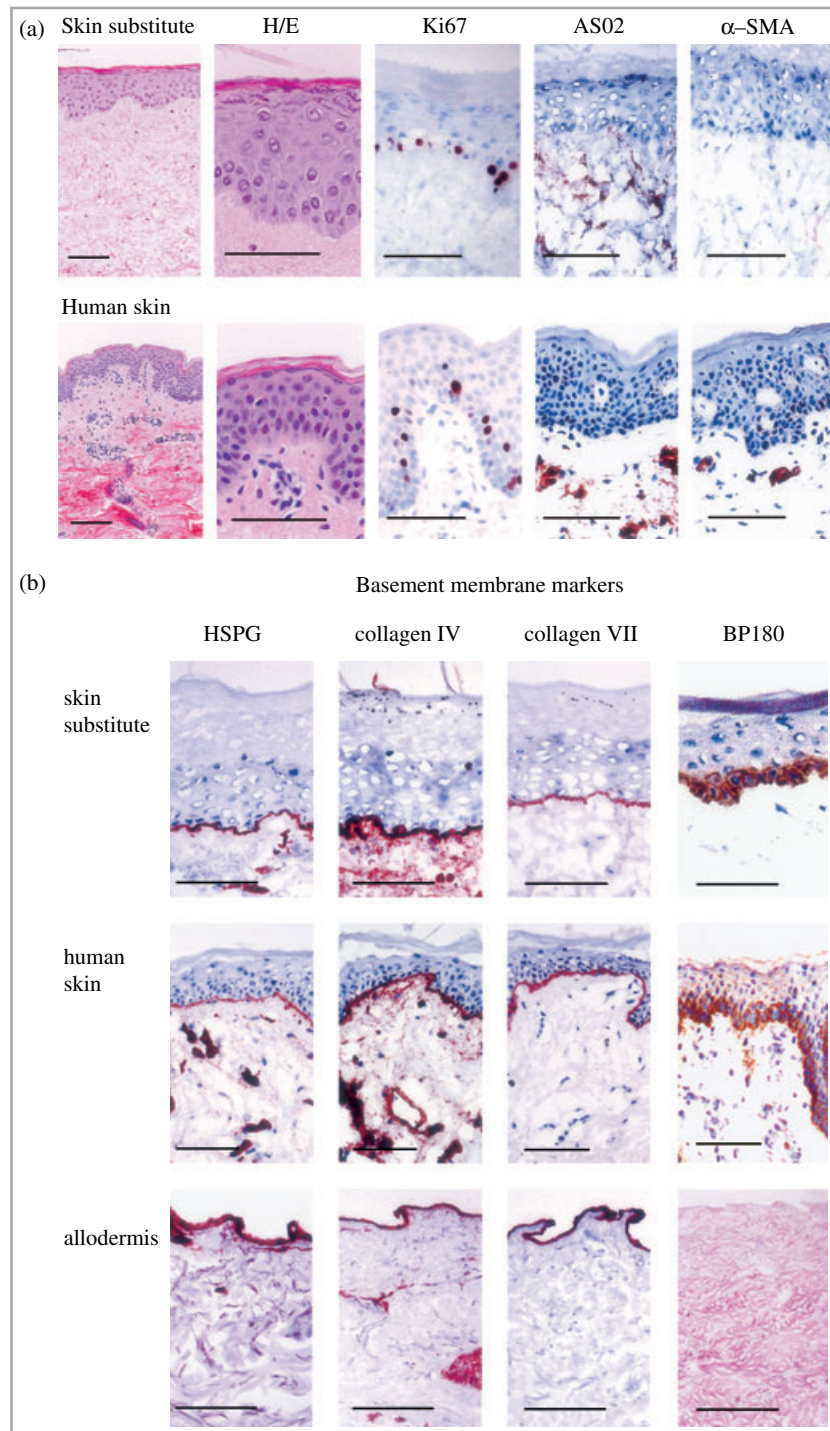


Fig 1. (Immuno-)histological comparison of the tissue-engineered skin substitute with healthy skin. (a) Haematoxylin and eosin (H/E: low power and higher power) staining of a cross-section of the newly formed epidermis shows that complete epidermal differentiation has occurred. Immunohistochemical staining with: anti-Ki67 shows that all dividing keratinocytes are located in the basal layer; anti-AS02 shows fibroblasts in the dermis; and anti- α -smooth muscle actin (SMA) shows the absence of myofibroblasts. (b) Analysis of the basement membrane zone: immunohistochemical staining of sections of the skin substitute, healthy skin, and acellular allodermis used to construct the skin substitute is shown. HSPG, heparan sulphate proteoglycan. Skin substitutes were harvested after 3 weeks of culture. (Immuno-)histology was part of the quality control of skin substitutes applied to patients. A representative skin substitute is shown for patient 3. Healthy skin (control) was obtained from adult abdominal skin from nonincluded donors. Allodermis is the acellular dermal matrix used for constructing the skin substitute. Bars = 100 μ m.

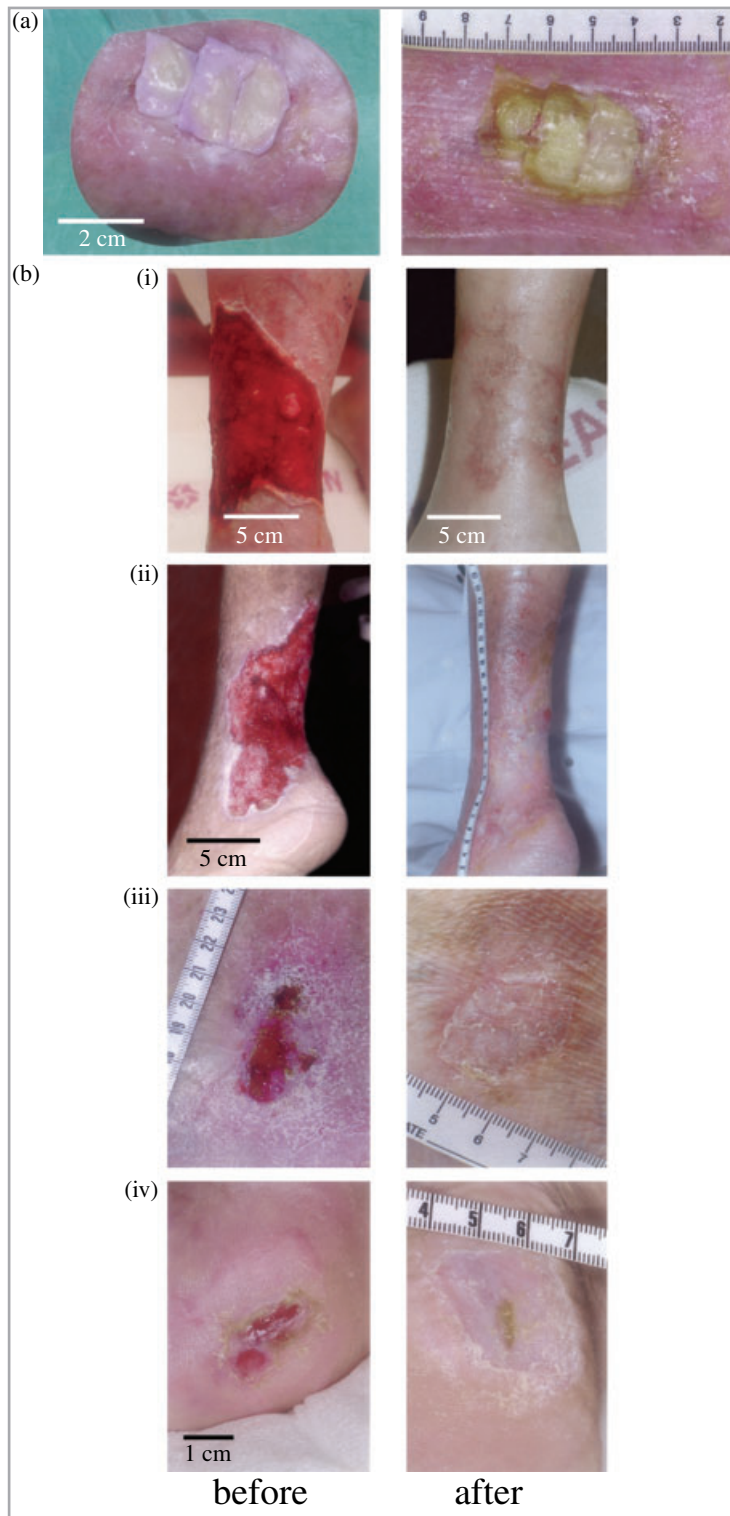


Fig 2. Clinical application. Skin substitutes were applied to previously nonresponding leg ulcers. (a) Application of the skin substitute (left) and 1 week after application (right) is shown. Viable epidermis is seen growing from the skin substitute on to the wound margins in order to close the wound completely. (b) Application of the skin substitute results in ulcer healing. For patient details see Tables 1 and 2 and Materials and methods. Patients are shown before and after complete healing: (i) patient 14, 5 months; (ii) patient 6, 11 months; (iii) patient 5, 6 months; (iv) patient 10, 2 weeks.

Discussion

We describe an autologous tissue-engineered skin substitute that visibly resembles a native autograft. This skin substitute proved to be successful in healing chronic wounds and is the first tissue-engineered, autologous, full-thickness skin substitute of entirely human origin to be used for wound healing.

Construction of the skin substitute is based on the fact that fibroblasts stimulate epidermal proliferation, differentiation and migration across the intact basement membrane, while epidermal cells stimulate fibroblast proliferation and migration into the dermis.¹⁵⁻¹⁷ Therefore, *in vitro*, cross-talk occurs between the different cell types in order to form the natural tissue architecture of the skin. In order to construct the skin

substitute an intact epithelial sheet was used from a punch biopsy. This approach was favoured above making a single-cell suspension of keratinocytes as it maintains epidermal integrity, and also enables extremely small amounts of donor skin to be cultured and expanded *in vitro*. Use of a single biopsy is also of importance when considering logistics (transport to/from the laboratory and number of patient visits to the hospital) and the burden to the patient involved. The method proved to be very reliable, with a 97% success rate in culturing the biopsies independent of the age of the patient. Our method of using the intact epidermal sheet and allowing the migration of primary fibroblasts directly into the allodermis, in addition to enabling the skin substitute to be constructed from a single biopsy, requires remarkably few technical procedures and in this way varies from previously reported methods for constructing skin substitutes of entirely human origin.^{18,19} For example, similar to us, the groups of El-Ghalbzouri and Chakrabarty both use human allodermis as a matrix. However, time-consuming methods involving isolation of single-cell suspensions, prior expansion of keratinocytes and fibroblasts, multiple-step procedures and multiple biopsies are involved in constructing the skin substitute. Another example of a skin substitute which is entirely of human origin involves seeding keratinocytes (passage 3) on to fibroblast sheets grown to confluency for 28 days (fibroblasts passage 5–9).²⁰ Again, logistical problems involving the cell culture are limiting factors in this study. None of these constructs has been used in a clinical setting for wound-healing procedures.

The skin substitute was directly applied to a wound in a simple one-step surgical procedure similar to that used when applying an autograft to a wound.²¹ Furthermore, only a single application was required. In all cases, whether the skin substitute was incorporated or not, wound healing was stimulated to such a degree that complete closure was achieved or a significant decrease in ulcer size was achieved. It is unknown whether the primary effect of the skin substitute is due to the continuous secretion of wound-healing factors, the direct take of the skin substitute resulting in wound closure, or a combination of both. However, the benefits of the single application of a living skin substitute outweigh frequent repeated topical applications of wound-healing factors (which become readily inactivated by proteases in the wound bed). The ability of the skin substitute to strongly stimulate granulation tissue was also confirmed after application of the skin substitute to three acute surgical wounds. The skin substitute was not rejected. However, hypergranulation occurred in all three cases (Gibbs *et al.*, unpublished data). This indicates that a less active construct suitable for optimally closing acute wounds without resulting in hypergranulation needs to be developed. This is currently under investigation.

Studies involving application to ulcers of native autografts, Apligraf® (allogeneic reconstructed epidermis on fibroblast-populated bovine collagen gel; Organogenesis, Canton, MA, U.S.A.) and Dermagraft® (allogeneic fibroblasts seeded on to a bioabsorbable polyglactin mesh scaffold; currently no longer available), have been performed. Application of autografts

(meshed or punch grafts) requires considerably more donor skin than application of a skin substitute. Furthermore, no evidence of a significant benefit to the patient has been shown for autografts over conventional therapies when used in chronic wound closure.²² Apligraf and Dermagraft both improve the time for wound healing compared with control groups receiving only compression therapy. However, repeated applications of both Apligraf and Dermagraft are generally required.^{7,23} A possible reason for the requirement of a repeated application may be that allogeneic cells are used which only remain viable for 4–8 weeks.²⁴ Trials using Apligraf and Dermagraft showed variable healing success rates ranging from 30% to 71% (Dermagraft) and 13% to 80% (Apligraf).^{7,12,23,25–28}

In our study, the patient to be treated with the skin substitute had a preinclusion period of 8 weeks in which weekly treatment in the outpatient department together with continuous compression therapy/application of ointments and dressings failed to induce a healing tendency (ulcer duration 0.5–50 years). This promising study is a prospective noncontrolled case series, and a randomized controlled study is now under way. The effect of VAC therapy alone compared with VAC therapy followed by application of the skin substitute has not been investigated in detail in our study and also warrants further investigation. VAC therapy removed debris from the wound bed and stimulated granulation tissue formation. We did not observe any difference between larger or smaller wounds after VAC therapy. It is not possible to make a direct comparison of our present results with those obtained using Apligraf or Dermagraft as patient groups vary with regard to control groups, type, size and duration of ulcer and the type of wound bed preparation.^{7,12,23,25–28}

Although autologous skin substitutes will not become an 'off-the-shelf' solution to wound healing, they do have the potential to become the most effective wound-healing product. The logistics around the application of these skin substitutes, which include the tissue culture period and transport, are items which require good organization but are not limiting as patients requiring these advanced therapies are generally frequent visitors to outpatient departments where elective (planned) therapy occurs rather than emergency treatments. Notably, even though the number of patients in our study is small, the results indicate that the skin substitute has a great ability to heal previously inert chronic wounds. A follow-up study will determine the extent of ulcer recurrence and scar formation. Research in the future will be aimed at developing skin substitutes which are also suitable for the treatment of larger diabetic, decubitus, venous and combined arterio(lo)sclerotic/venous ulcers as well as burns and acute surgical wounds.

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