Primary keratinocyte cell culture on EpiGen membranes for autologous skin grafts in paediatric burn patients

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Cultured epithelial autografts have been shown to be an effective permanent skin replacement for major burn injuries and have proved life-saving when insufficient donor skin has been available. Several membrane systems have been developed that facilitate the transfer of cultured cells on to the recipient. The aim of the study reported here was to test the effectiveness of EpiGen, a synthetic polymer membrane, as a cell culture support matrix for the transplantation of cultured autografts. Skin biopsies were obtained from 22 paediatric burn patients with an affected total body surface area of between 7% and 80%. Basal keratinocytes were harvested from the dermal/epidermal junction and cultured in a collagen 1-coated flask in modified Green's medium. After two passages, isolated keratinocytes were grown on EpiGen membranes until semi-confluent. Wound beds were excised and covered with widely (1:3) meshed split skin grafts. Membranes were grafted with the basal cell layer directed against the wound bed. Unseeded membranes were applied and served as controls. Wounds were dressed and closed appropriately. Grafts were regularly inspected for 'take' and the membranes were removed 10 days after application. Seven patients had to be excluded from the study. Cell culture results of the remaining 15 patients showed excellent cell growth and expansion on EpiGen membranes within a mean culture time of 2.6 days post membrane seeding. The membranes facilitated easy transfer of cultures onto the recipient. A mean keratinocyte graft 'take' of 95% and a mean control graft 'take' of 90% were recorded at the time of membrane removal. The mean level of clinically evident re-epithelialization on keratinocyte grafted areas in recipients was 87% as opposed to 60% in the unseeded control areas.

Introduction

The mortality rate of patients with extensive burn injuries remains high when more than 70% of total body surface area is affected. Excision of the burnt area with immediate autologous skin cover is the most important factor to reduce mortality. In these patients, the lack of adequate donor sites for autograft procurement represents a major problem. To overcome this deficiency, various methods have been developed involving the use of allografts, biological and synthetic skin substitutes or cultured epithelial autografts. The last has become a recognized method of alternative skin replacement.[1,2] This treatment has resulted in successful permanent coverage of full thickness burn wounds and, in many cases, has proved life-saving when insufficient donor sites have been available.[3–7]

The concept of epithelial autografts was developed two centuries ago.[8,9] Continuous research over the following nine decades culminated in the first successful transplantation of autologous cultured keratinocytes in 1981.[10] Initial reports documented the application of cultured epithelial autografts (CEA) alone.[6,10–14] Several of these studies showed that CEA alone do not provide ideal permanent wound coverage.[6,12–16] The reported clinical 'take' was variable, prone to mechanical disruption and blister formation. However, permanent skin replacement could be achieved when CEA was used in combination with meshed split skin grafts[5,17] or allodermis.[3,18–20]

However, transfer of the cultured keratinocytes from the culture flask to the patient remains problematic, although improvements in this regard have been reported. Keratinocytes embedded in a fibrin glue suspension have successfully been transplanted in full thickness burns,[21–23] and delivery of an epithelial cell suspension via an aerosolization apparatus has been demonstrated in porcine models[24–27] and a clinical study.[28]

More recently, the introduction of keratinocyte membrane delivery systems has offered an alternative approach for the transplantation of cultured keratinocytes onto wound surfaces.[29–36] Although various delivery systems have been developed, the search for the ideal culture support and transfer matrix still continues. The work reported here is an attempt to evaluate the commercial membrane, EpiGen, as an alternative to available culture delivery techniques. If as successful as we believe they can be, EpiGen membranes should also be considered as primary cover for 'sprayed on' cultured epithelial autografts, as they have been reported to provide a moist wound healing environment and are impermeable to bacteria. The EpiGen products, developed by Smith & Nephew Group plc Ltd, are biocompatible, translucent, flexible polymer membranes. They are supplied in the form of inserts inside 48-mm circular, sterile dishes into which keratinocytes are transferred and cultured just prior to transplantation onto the recipient. In a pilot study, EpiGen membranes were shown to facilitate the culture and transfer of sub-confluent autologous keratinocytes to patients.[35] Unequivocal evidence of keratinocyte transfer from EpiGen membranes to the recipient has been documented in a porcine model.[36] For this purpose, retroviral transfer of a lacZ reporter gene was used to mark a proportion of cultured autologous porcine keratinocytes *in vitro*. Definitive proof of keratinocyte transfer was obtained as witnessed by macroscopic areas of blue-stained surface tissue on experimental, but not control, wounds. Furthermore, lacZ-marked cells were identified in deeper layers of epithelial tissue in cryo-sections through wound biopsies, demonstrating the efficiency of keratinocyte delivery using inverted membrane transfer.

Materials and methods

Patient study group

Twenty-two patients admitted to the burns unit at the Red Cross Children's War Memorial Hospital, Cape Town, were the subjects of this study. The patients' ages ranged from 11 months to 8 years, with a mean of 3.5 years. Partial and full skin thickness burns covered between 7% and 80% of total body surface area. In all, 27 skin biopsies were processed from these children. Two biopsies were obtained from three patients and three skin samples from one patient. Informed consent was obtained from all patients' parents or guardians.

Each child received standard burn wound care therapy as practised at our hospital. This entails daily hydrotherapy, topical therapy, and debridement of blisters and loose necrotic tissue. Antibiotic treatment was reserved for documented infections. Nutrition was maintained enterally. Fifteen skin biopsies were taken under general anaesthetic, seven were obtained within 3–7 days of patient admission and 8 samples between 10 and 20 days. In the majority of cases, biopsies were taken from the region of the thigh.

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Isolation and culture of primary keratinocytes

Partial thickness skin biopsies, approximately 2 × 4 cm in size, were obtained by means of a Davies dermatome (setting 3–4) from non-burnt areas. The samples were transported without delay to the laboratory in a sample jar containing sterile phosphate-buffered saline (PBS) without antibiotics. All biopsies were processed immediately unless stated otherwise. Keratinocytes were isolated according to a modification of the method of Rheinwald and Green.[37] Briefly, this entailed the following: the biopsy was cut into smaller fragments, approximately 0.5 cm^2 in size, and digested with 2.5% trypsin at 37°C in the shortest possible time. This varied from 30 minutes to two hours, depending on the thickness of the skin section. The fragments were rinsed in sterile PBS before the mechanical detachment of epidermis from dermis. Basal keratinocytes were harvested from the dermal/epidermal junction and pipetted into a solution of PBS containing 20% foetal bovine serum to arrest residual trypsin activity. The pooled cell suspension was centrifuged and the pellet resuspended in Green's medium, which included: insulin (5 mg/ml), hydrocortisone (0.2 mg/ml), cholera toxin (1×10^{-7} M), epidermal growth factor (10 μg/ml), triiodo-L-thyronine (2×10^{-6} M), transferrin (5 mg/ml), penicillin (5000 IU/ml), streptomycin (5 mg/ml), gentamycin (50 mg/ml), and 10% foetal bovine serum. For culture purposes, 40×10^5 cells were seeded into 75-cm^2 collagen 1-coated, vented tissue culture flasks containing Green's medium. Cells were cultured at 37°C in a 5% CO_2 atmosphere. The medium was changed 3 days later when most of the epidermal cells had attached. The cultures were examined regularly for signs of colony formation and trypsinized with 1x trypsin/EDTA once 70–80% colony confluency became apparent. The passaged cells were re-seeded into fresh flasks for secondary cultures, and the medium was changed at appropriate time intervals. When the secondary cultures had reached 70% confluency, trypsinization was repeated. This tertiary keratinocyte cell culture was then seeded onto sterile EpiGen membranes at a density of 30 000 cells per cm^2. After 2–5 days' incubation at 37°C in 5% CO_2, the keratinocytes on the membranes reached, on average, 50–60% confluency and were ready for autologous grafting after several washes in sterile PBS to remove residual culture medium.

For the identification of keratinocytes, cytocentrifuge preparations of the cells were fixed and stained with Rhodanile blue, a combination of Rhodamine B and Nile Blue first synthesized by MacConaill and Gurr.[38] This mixture of dyes results in red staining of keratinocytes due to the Rhodamine B component. Other well-known culture lines of other than epidermal origin such as 3T3, HeLa cells and human diploid fibroblasts have been reported to give a blue colour after staining with Rhodanile blue.[39]

The viability of the cells harvested initially and at subsequent passages was determined by standard trypan blue exclusion using light microscopy.

Table 1. Summary of clinical data on patients and/or samples excluded from the trial.

Patient number	Exclusion criteria	Age (years; m = months)	Type of burn	TBSA* (%)	Degree	Past medical history
1	Died	4	Fire	60	Full	Unknown
2	Repeat graft	4	Fire	55	Full	Nil
3	Died	5	Fire	80	Full	Nil
4	Graft deviation	5	Fire	50	Full	Nil
5	Graft deviation	5	Hot water	35	Partial full	Nil
6	Contamination	5	Fire	50	Full	Nil
7	Biopsy failure	5	Fire	50	Full	Nil
8	Contamination	11 m	Hot water	21	Partial	Nil
9	Contamination	6	Hot water	21	Full	No record
10	Contamination	6	Hot water	21	Full	No record
11	Died	7	Fire	70	Partial full	TB
12	Culture failure	6	Fire	22	Partial full	Nil

*TBSA, total body surface area.

Table 2. Summary of clinical data on patients included in the trial.

Patient number	Age (years; m = months)	Type of burn	TBSA* (%)	Degree	Past medical history
1	4	Fire	55	Full	Nil
2	18 m	Hot water	10	Partial–full	Gastroenteritis
3	1	Hot water	7	Superficial–partial	Nil
4	15 m	Hot water	22	Superficial–partial	Not recorded
5	2 yr 6 m	Hot water	30	Partial–full	Nil
6	3	Fire	15	Full	Nil
7	11 m	Hot water	10	Partial–full	Gastroenteritis
8	2	Hot water	17	Partial–full	Nil
9	7	Fire	10	Partial–full	Nil
10	2	Fire	20	Full	Nil
11	1	Hot water	18	Full	Nil
12	8	Hot water	16	Superficial–partial	Nil
13	5	Fire	28	Partial–full	Nil
14	6	Fire	22	Partial–full	Nil
15	3	Hot food	17	Partial–full	Not recorded

*TBSA, total body surface area.

Wound bed preparation and grafting of cultured keratinocytes

In theatre, the wound beds were excised and covered with widely meshed (1:3) split skin grafts. The cultured autografts were then overlaid onto the grafted areas with the basal cell layer directed against the wound bed. In each instance an unseeded EpiGen membrane, placed adjacent to a seeded membrane, served as a control. The EpiGen membranes were clipped or sutured in place and covered with the non-adhesive, transparent, soft silicone wound contact layer Mepitel (Mölnlycke Health Care AB, Sweden) and dressed with topical mupirocin. A closed wound technique was used. Inspections and photographic capture of graft 'take' and re-epithelialization were carried out on alternate days at dressing changes and the membranes were removed on day 10.

Results

Skin biopsies

Although keratinocytes from 20 biopsies could successfully be seeded onto EpiGen membranes, results of 12 of the 27 biopsies had to be excluded from the clinical study (Table 1). The reasons for this exclusion were as follows: three patients with 60%, 80% and 70% total body surface area died shortly after their initial skin harvest and four biopsies were contaminated due to persistent patient infection with *Pseudomonas* species, yeast, or *Klebsiella pneumoniae*. In two children there was a deviation from the grafting protocol. In one child the EpiGen membranes were

Table 3. Keratinocyte isolation and culture results.

Patient number	Biopsy site	Biopsy storage	Biopsy thickness*	Trypsin time (min)	Confluency at passage 1 (%/day)	Confluency at passage 2 (%/day)	Confluency on EpiGen
1	Arm	None	Medium	105	80/7	80/2	50/4
2	Thigh	None	Very thin	30	80/6	70/2	50/3
3	Buttock	None	Very thin	30	80/6	80/2	60/3
4	Thigh	None	Very thin	30	60/6	90/2	90/2
5	Thigh	None	Medium	90	60/6	80/2	60/2
6	Thigh	o/n, 4°C	Thick	120	90/6	80/3	50/2
7	Thigh	None	Thin	75	80/5	70/3	50/2
8	Back	None	Medium	90	80/5	80/3	60/3
9	Thigh	None	Medium	105	80/5	80/3	50/3
10	Thigh	o/n, 4°C	Medium	90	80/5	70/3	50/4
11	Thigh	None	Medium	75	90/5	70/2	60/2
12	Thigh	None	Thin	45	70/5	70/3	60/3
13	Thigh	None	Thin	60	80/4	70/2	40/3
14	Arm	None	Medium	90	80/4	90/3	60/2
15	Thigh	None	Medium	60	70/4	80/3	50/2

*Thin, epidermis and basal layer; medium, epidermis and soft dermis; thick, epidermis and mid dermis; o/n, overnight.

applied directly onto the excised wound bed as insufficient donor sites were available for autologous meshed grafts. In the second child the seeded membranes were applied in the absence of a control membrane. In addition, insufficient cells could be harvested from one biopsy and the cells of another culture failed to expand in sufficient numbers. One patient underwent two successful grafts, of which only one is reported in the final results. The clinical data of the remaining 15 patients finally included in the study are summarized in Table 2.

Isolation and cultivation of keratinocytes

Some of the parameters that were recorded in the laboratory for the purpose of isolating and cultivating autologous keratinocytes derived from the remaining 15 patients are shown in Table 3. The majority of the biopsies were excised from the thigh and processed immediately. The thickness of the dermatome sections varied. This had a direct effect on the timing of the enzymatic dissociation of epidermis from dermis, which ranged from 30 minutes in the very thin sections to two hours in the thick sections. In most instances cell suspensions showed a brown coloration indicating the concomitant harvesting of melanocytes. The time for primary cultures to reach 70–80% colony confluency before the first passage was not consistent but varied from 4 to 7 days. This differed from the time required by all cultures to reach similar cell confluencies after the first passage, that is, 2–3 days. Microscopic examination of the cytocentrifuge preparations of epidermal cells harvested from patient 1 at the first and second passages showed red-stained cells, which confirmed that the isolated cells were of keratinocyte origin. Examination of cell suspensions by trypan blue exclusion at initial harvests and at passages yielded greater than 85% and 90% viable cells, respectively, in each instance.

Keratinocytes seeded onto EpiGen membranes displayed firm adherence to the membranes. Upon microscopic examination of the cultures after overnight incubation, signs of keratinocyte colony formation were already apparent. The expansion of these colonies proceeded rapidly and 50–60% confluency was evident within 2–4 days after membrane seeding. PBS rinses of the cultures on the EpiGen, to remove residual culture medium, did not result in the detachment of cells from the support matrix. All cultures grown on 18-cm^2 membranes showed a refractile, sub-confluent cell layer of normal cell morphology under phase contrast microscopy prior to membrane grafting. The period taken from the receipt of the biopsy to the time when keratinocytes cultured on EpiGen membranes were ready for grafting was 9–13 days.

Graft assessment

Graft 'take' was assessed visually on alternate days at the time of dressing changes, starting on day 2. This continued up to day 10, by which time the membranes were removed. In a large proportion of cases the cell-seeded membranes started to detach from the wound bed by day 6 or day 8, which indicated that the transfer of the keratinocytes from the membranes onto the burn wound was complete. A visual graft assessment after removal of the EpiGen membranes on day 10 showed 100% keratinocyte graft 'take' in 13 patients and 70% in one patient (Table 4). The mean keratinocyte graft 'take' in the patients was 95%. A mean level of 87% re-epithelialization was clinically evident in patients who had received CEA. The mean percentage 'take' in MSSG, covered with EpiGen membranes, was 90%. In these areas the mean percentage re-epithelialization varied considerably, ranging from 15 to 100% with a mean of 60%. Patient 10 experienced a keratinocyte and control graft loss due to infection with *Pseudomonas*. Poor re-epithelialization was apparent in patient 13 in both grafts and the membranes had to be removed on day 9 due to an underlying *Pseudomonas* infection. Figure 1A

Table 4. Graft 'take' and re-epithelialization on day 10.

	EPI[a] + CEA[b] + MSSG[c]		EPI + MSSG	
Patient number	Graft 'take'[d] (%)	Re-epithelialization[e] (%)	Graft 'take' (%)	Re-epithelialization (%)
1	70	100	100	40
2	100	98	100	100
3	100	100	85	15
4	100	100	100	40
5	100	85	80	60
6	100	100	50	50
7	100	100	100	25
8	100	100	100	90
9	100	100	100	100
10	50	0	30	0
11	100	100	100	100
12	100	100	100	30
13	100	25	100	50
14	100	100	100	100
15	100	100	100	100

a, EpiGen membranes; b, cultured epithelial autograft; c, meshed split skin graft; d, mean level of adherence of matrix-supported keratinocytes to recipient area; e, mean level of re-epithelialization.

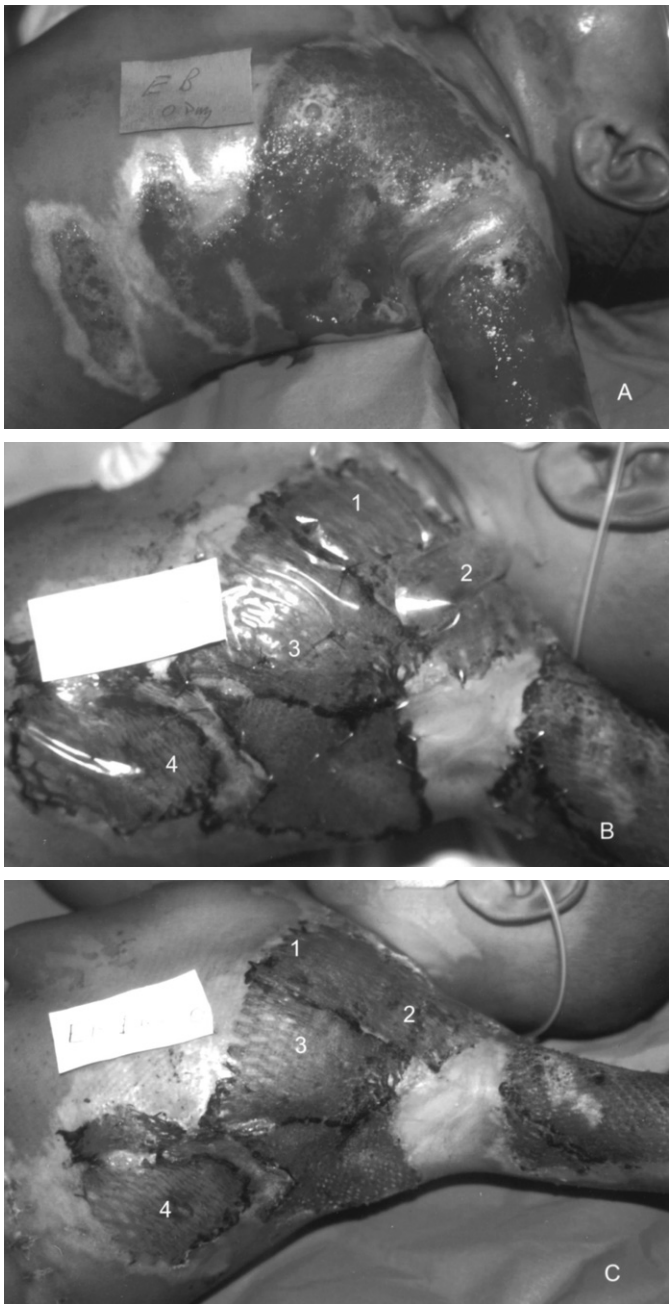

Fig.1. **A**, Burn wound covered by granulation tissue on day 0; **B**, application of cultured epithelial autografts + EpiGen + meshed split skin grafts (areas 1, 2, 3) and control (area 4) on day 8; **C**, 100% re-epithelialization of cultured epithelial autografts + EpiGen + meshed split skin grafts (areas 1, 2, 3) and 25% re-epithelialization of control (area 4) on day 10.

shows a partial to full thickness burn wound on day 0, covered by granulation tissue. This patient received three cell-seeded EpiGen membranes and one unseeded control membrane marked 1, 2, 3 and 4, respectively (Fig. 1B). At the time of membrane removal on day 10, 100% graft 'take' and complete re-epithelialization was evident in the areas covered by CEA, but only 25% re-epithelialization occurred in the control area (Fig. 1C).

Discussion

The ability to culture autologous basal keratinocytes is of great importance to the clinician in the treatment of burn victims. A review of current practices revealed a variety of deviations from original protocols published in the literature. These differences, at least in part, are consequences of continued attempts, by researchers and clinicians alike, to improve existing cultivation techniques and final graft transplantation procedures.

Since biopsy surface area, not depth, is an important factor in ensuring that sufficient cells are harvested in the initial stage of isolating keratinocytes, dermatome sections were used in preference to punch biopsies. An additional advantage of the dermatome sections was the absence of adipose tissue, which obviated the need to trim the sections prior to enzymatic dissociation of epidermis from dermis. No noticeable difference in cell growth, cell expansion, colony confluency or total culture time was apparent in samples derived from the different sites of the body. Enzymatic treatment of the dermatome sections in the shortest possible time appeared to be a critical step, not only for ease of mechanical separation but also for the ability of keratinocytes to form expanding colonies during subsequent culture.

Keratinocytes derived from 15 patients showed a difference in the number of days to reach sub-confluency. This was apparent only in the initial stages of their culture, prior to the first passage, and varied from 4 to 7 days. This difference did not appear to be age related as keratinocytes from the youngest patient (11 months) and the oldest (8 years) displayed the same time requirement of 5 days. Given the fact that culture conditions were the same for all keratinocytes, it would be reasonable to assume that this difference might be due to some intrinsic factor(s) which have an influence on the growth and expansion of the cells. One such contributing factor could be small variations in the lag period amongst the primary cultures. It is also possible that the number and clonal type of colony forming cells, present in the initial cell pools, might differ, which would contribute to small variations in the appearance of colony sub-confluency.[40]

One of the many obstacles in the culturing of keratinocytes over decades has been the appropriate procedure of transferring the cultured cell sheet from the tissue culture flask onto the wound bed of the recipient. To circumvent this problem, synthetic membranes have been developed which not only support cell growth but are suitable for the transfer of the cultured cells onto the wound bed.

In this study, EpiGen membranes were used as a support matrix for both the cultivation and grafting of paediatric keratinocytes. Cells seeded onto the membranes after the second passage actively proliferated into healthy, semi-confluent subcultures. Prior to grafting, the membranes could be rinsed with PBS without any loss of or damage to the cells. Transport of the cultured autograft to the theatre could be done in the original, sterile EpiGen culture dishes and thereby avoided possible contamination or loss of the graft. In the operating theatre the pliable membranes with adhering keratinocytes could be detached from the base of the dishes with great ease and clipped or sutured onto the prepared wound bed without any loss of cells. One limiting factor was the observed effect of membrane buckling in uneven or over mobile, flexible areas.

The mean culture time of the paediatric keratinocytes was 10.5 days, which is relatively short. This falls within the time that is required to stabilize and to prepare a patient for surgical eschar excision and grafting. The observed mean percentage graft 'take' was similar in CEA and controls. It could be argued that the 'take' in both instances was solely due to the underlying meshed split skin grafts in both grafts. However, the effective contribution of the keratinocytes to the wound repair process can clearly be seen by a marked increase and improvement in the re-epithelialization in CEA grafted areas compared to unseeded control

areas. A full clinical account of the wound repair process, compared to unseeded membrane and meshed split skin graft alone, is in preparation. This will include assessments of pigmentation, vascularity, pliability and scar height using the Burn Scar Index, also known as the Vancouver Scar Scale.[41]

Grafting of cultured, autologous keratinocytes offers several, distinct advantages. CEA provide an additional source of autologous skin for large burns. In addition, small donor areas can be expanded to cover larger recipient areas. This is especially important when donor areas become a limiting factor. Ease of graft application is facilitated by cultures supported on EpiGen membranes and the transplanting of autologous cells leads to improved cosmetic appearance of the grafted area.

Results of this study provide evidence of the effectiveness of EpiGen membranes as a cell culture support and transfer matrix for the transplantation of cultured autografts in paediatric burn patients. In addition, these membranes can be considered as primary cover for 'sprayed on' cultured epithelial autografts.

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