In three previous articles, we have described a planned two-stage strategy to negate the need for skin grafts, specifically in those subjects with major burn injury where donor sites are scarce.1–3 This strategy requires two different three-dimensional products, which we have created based on the NovoSorb™ biodegradable polyurethane platform. The first is a biodegradable temporizing matrix (BTM, a 2-mm thick biodegradable polyurethane foam matrix) to which a 150 µm nonbiodegradable polyurethane film is bonded to seal the superficial surface. This has been designed for application, following deep burn excision, into the resulting wound, to integrate into and temporize the wound, and to minimize contraction and resist infection. As a dermal template, it has been designed to mirror the functions of materials such as Integra™ dermal regeneration template. The second is a composite cultured “skin” (CCS)—autologous fibroblasts seeded and cultured into a 1-mm unsealed NovoSorb™ foam matrix and allowed to lay down collagen before receiving autologous keratinocyte seeding on its superficial surface. The resulting cultured composite takes ~21 days to prepare using a perfusion culture system. When ready, the BTM seal is removed, the “neodermal” surface refreshed by dermabrasion, and the composite applied. Our previous articles demonstrate the early assessment of the NovoSorb™ material and the subsequent investigation of the BTM product developed from it. This article describes our effort to close the integrated
BTM with the CCS product and its potential in wound closure, aiming to prove the concept of the planned two-stage strategy.

The use of tissue-engineered skin substitutes is becoming widespread in acute burn management. Compilations of different structural forms and composites have been developed; however, none has proven ideal, marred by the potential for infection, cost, aesthetics, or production resources.

The genesis of tissue engineering in the field of skin was in 1975 when Rheinwald and Green pioneered the technique of keratinocyte culture allowing the development of epidermal substitutes.4 Over the past 35 years, two clinically employed variants of keratinocyte-alone products have emerged: cultured epithelial autograft (CEA) sheets and preconfluent keratinocyte suspensions.5,6 Therefore, before commencement of the composite study, it was essential to exclude the possibility that keratinocyte application alone, as suspensions or sheets, early and at low (clinically producible) seeding densities, might allow epidermal formation over the integrated BTM. This, however, was not demonstrable. No central keratinocyte “take” or subsequent epithelialization was evident. The integrated BTM was unable to support CEA in the form of keratinocyte sheets or suspensions, and as a result the current study proceeded.

Cultured skin substitutes exist as adjuncts to traditional therapies in the management of large TBSA burns. There are commercially available substitutes on the market, such as Apligraf®, but availability is limited, and such materials may contain allogeneic components.7 Most cultured substitutes seem to be produced by individual units around the world for their own use. Collagen,8,9 collagen-glycosaminoglycan,10-14 plasma,15,16 polyethylene glycol terephthalate,17 polyglycolic acid18 poly(lactic-co-glycolic acid),19 polygalactin,20 and amniotic membrane21 have all been employed as the dermal scaffold with the addition of keratinocytes and fibroblasts. However, to date, none has been able to successfully eliminate the need for autograft in large burns. The aim of this study was, therefore, to assess the integrated BTM’s ability to support autologous cultured composite “skin” (CCS) in its ability to effect wound closure.

METHODS

Animals

This study was conducted in the large animal research imaging facility of SA Pathology and approved by the local animal ethics committee (AEC#56/11). Three large white/landrace cross domestic pigs (Sus scrofa) initially weighing 22.2 to 33 kg were acclimatized for 1 week before study commencement. Housing and animal care were provided in accordance with National Health and Medical Research Council guidelines. All animals were fasted overnight before surgery, and subsequent dressing changes and measurements were performed under general anaesthesia. Co-Amoxyclov (Clavulox, 2.5 ml) was given intramuscularly for 3 days postsurgery and again posttreatment. Additional antibiotics were administered if any signs suggesting infection were observed. Weight, length, and girth were recorded at each time point to monitor animal growth.

Biodegradable Polyurethane Matrices

Matrices were provided by PolyNovo Biomaterials Pty Ltd (Port Melbourne, Victoria, Australia) as 10 × 10 cm pieces, sterilized by γ-irradiation and dry-packed. The BTM is a 2-mm thick foam matrix with a 150-µm thick, nonbiodegradable, microporous polyurethane “sealing” membrane, bonded to its superficial surface. The scaffold for the production of the CCS was a 1-mm thick, unsealed foam matrix.

Porcine Wound Model

The surgical and dressing protocols for this study were performed as previously described2,3 with minor amendments. Four 8 × 8 cm sites were designed on the flanks of the pig. Split-thickness skin grafts (12/1000th of an inch) were taken from each animal to provide autologous components for keratinocyte cell culture. The donor site wounds were then deepened to the panniculus adiposus and 2-mm thick sealed BTM polymers were implanted affixed with surgical staples.

Treatment Allocations

The treatment sites in this study were

(1 and 2) a sealed BTM with CCS applied after BTM integration at day 28
(3) a sealed BTM with split-thickness skin graft applied at day 28
(4) a fresh wound (to panniculus adiposus) created at day 28 with CCS applied

Two of the BTM-treated sites received CCS at day 28 and the other a split-thickness skin graft taken from area four (initially untreated). This fourth (donor) site was then deepened to a full-thickness wound and grafted with a CCS only on day 28.

Isolation and Culture of Cells for Composite Creation

Split-thickness skin biopsies harvested from each pig were processed for cell isolation. Skin samples
(~8 × 8 cm) were washed thoroughly in multiple povidone–iodine, gentamicin, and phosphate buffer saline solutions, cut into small pieces and incubated overnight in Dispase II (6 mg/ml) at 4°C. The epidermis was separated and processed for keratinocytes.

Basal keratinocytes were isolated by trypsinizing the epidermal sheets with 0.05% Trypsin-EDTA (Sigma, St. Louis, MO) with gentle agitation for 5 minutes. This solution was then quenched with equal volumes of Soybean trypsin inhibitor (Sigma, St. Louis, MO). Keratinocytes were cocultured with irradiated Swiss albino (iSA3T3) fibroblasts in keratinocyte growth media, SEL-KGM-1% FBS (based on Rheinwald and Green’s original media). The dermal component of the biopsy was required to isolate the fibroblasts. It was cut into smaller (0.3 cm²) pieces and further digested with Collagenase I (3 mg/ml) at 37°C with gentle agitation until the dermal pieces were virtually digested (3–5 hours depending on the thickness of the dermis). After several washes, the cells were centrifuged and cell counts performed. Fibroblasts were cultured in DMEM-10% FBS medium supplemented with antibiotic/antimycotic (Sigma, St. Louis, MO). Cells were incubated at 37°C with 5% CO₂ and the medium changed every 2 to 3 days.

**Generation of CCS**

A plasma gel was developed to minimize cell loss through the composite polymer matrix structure and allow attachment to the culture vessel. This involved the use of one bag (265 ml) of blood, aseptically collected from the femoral artery of each pig, for autologous plasma and thrombin isolation. Aliquots of the plasma and thrombin were frozen for composition manufacture. A 1-mm unsealed foam matrix was used for the basis of the CCS. The matrices were pre-soaked in plasma before the addition of cells resuspended in autologous thrombin, forming the plasma gel-incorporated matrix.

Four 10 × 10 cm fibroblast composite gels were established in a large square 20 × 20 cm Petri dish (Corning Inc., NY) with 4 × 10⁴ cells per cm². The fibroblast-containing composites were allowed to “set” for 10 minutes at ambient temperature before being transferred to an incubator. Each dish was connected to a perfusion culture system (in-house) and media (DMEM-10% FBS) pumped in at 5 ml/minute (total 500 ml per dish).

After culture, keratinocytes (passage 1) were harvested for application onto the fibroblast-foam composite by differential trypsinization to remove residual iSA3T3 fibroblasts. Cells were uniformly added to the surface of the foam in a thrombin solution at 2.5 × 10⁶ per cm². Again, dishes were allowed to settle for 10 minutes before being transferred to an incubator for 20 minutes before media (SEL-KGM 5% FBS) was pumped in at 5 ml/minute. Media was changed every 2 to 3 days until composite application at 28 days postsurgery. For transplantation, media was drained from the culture vessel, and the CCS was transported to the theater for application.

One 10 × 10 cm composite was randomly assigned as a “spare” for serial analysis during the culture period. At different time points, biopsies from this sample were stained with viability dyes (Calcein/Ethidium, Sigma, St. Louis, MO) and analyzed using the Nikon confocal microscope. Light micrographs were also obtained during media changes using an Olympus C-5060 digital camera and microscope lens adapter.

**CCS Application**

The BTM treatment sites were delaminated of residual seal and dermabraded with a diamond burr to refresh the superficial surface of the wound, preparing them for composite application (Figure 1A). The composites were carefully applied (Figure 1B), trimmed to size (Figure 1C), and affixed with surgical steel staples (Figure 1D). An additional Mepitel™ piece, precut to each individual treatment area, was affixed rostrally with staples to minimize shear of the composites. The first dressing change allowed retention of this “under-dressing.” A large Mepitel™ (20 × 20 cm) dressing then covered all sites and standard procedures followed. Dressing changes occurred twice a week.

**Wound Assessments**

Wounds were cleaned, dressed, and visually assessed for infection, matrix integration, and reepithelialization. For macroscopic evaluation, digital photographs were taken with a Canon EOS550D SLR digital camera. Length, girth, and weight were measured to monitor pig condition. Wound areas were measured using the Visitrak™ system (Smith & Nephew Ltd., Hull, UK), and evaporative water loss was assessed using a Vapometer (Delfin Technologies Ltd., Helsinki, Finland). To minimize any disruption of the treatment site, tracings and readings were not obtained on day 3 after treatment.

**Histological Analysis**

Punch biopsies were obtained for histological assessment, and large, postmortem, full-thickness excision biopsies were collected at necropsy on day 42 postsurgery. These samples were fixed in neutral-buffered
formalin. A number of staining methods (Hematoxylin and Eosin [H&E], Periodic acid-Schiff, Immunohistochemical and fluorescence [BovK, 1:500; Dako, Z0622] for keratin) were employed to visualize and confirm the quality of reepithelialization, basement membrane formation, granulation tissue formation, fibroblast influx, in-growth of blood vessels, and collagen deposition. The histology slides were reviewed by an independent pathologist.

RESULTS

BTM Before CCS Application
At initial surgery, sealed BTMs were applied to the three treatment areas with seal delamination scheduled for day 28 postapplication. However, in a number of sites, seal delamination occurred before day 28, with partial seal removal and varying degrees of seal fragmentation with or without superficial granulation. Only two sites maintained a seal that could be delaminated in one whole piece on day 28. Because of the sealing shortfalls, the wounds had contracted to 50% of their original size (mean, 50.5%). As proof of concept of CCS take over integrated BTM foam was the only outcome sought in this study, application of the CCSs to the treatment areas were selective and the split-thickness skin grafts, taken from the fourth site, were applied to the smallest residual integrated BTM wound.

BTM With STSG
Partial STSG take was observed in one of the three pigs with the remaining grafts completely successful (100% take). They appeared normal and healthy, vascularizing early and maintaining wound size from application to end point. Histological analysis

showed the engraftment of normal STSG over the integrated 2-mm BTM (as previously shown).2,3

BTM With CCS
CCSs were easy to manipulate, apply, trim, and affix with steel staples. They conformed to the wound area and were flush to the wound edge (Figure 1C and D). The CCS was easily cut to size and no tearing was observed on their subsequent application with staples.

Proof of Concept Achieved
Successful “take” of the CCS on an integrated 2-mm BTM wound. Random punch biopsies taken throughout the study indicated that epithelium was present from day 3 postapplication.

Of the CCS sites, clinically four did not “take” and were removed on day 7 postapplication; however, on removal there appeared to be a small amount of residual foam on the surface indicating that the deeper component of the CCS had adhered to the wound. The two sites that demonstrated the proof of concept with successful “take” of the CCS on an integrated 2-mm BTM wound were from pig 2 (Figures 2 and 3). Clinical progression from day 3 postapplication to day 14 is shown in Figure 2A through C and Figure 3A through D, displaying CCS vascularization and integration by day 10. Clinically, these wounds displayed a desiccated appearance of the superficial surface polymer matrix, forming a stiff carapace over the underlying wound surface, which, when lifted, showed several areas of epithelium below (Figure 2B through D).

Furthermore, histological analysis demonstrated a well-developed epithelium integrated around and within the foam at day 7, 10, and 14 (Figure 2E through G). The integration of the 2-mm BTM on subcutaneous fat with the superficial 1-mm CCS “take” was evident on histological analysis (Figure 3E); however, the degree of 2-mm BTM “take” varied as discussed earlier. A defined basement membrane was present by day 7 (Figure 3F), and the presence of a thick keratinized layer above the epithelium was confirmed by staining with BovK (Figure 3G). The degree of integration of the 1-mm CCS also varied. There were areas where no CCS was visible, and the polymer had apparently “shed” from the healed surface, after depositing cells, thus acting as a delivery vehicle (Figure 2H). Partial CCS integration was exhibited in some wounds, with a deep layer of integrated CCS with surrounding epithelium and a superficial unintegrated matrix layer, or complete integration (“take”), where the entire CCS was surrounded and incorporated within an epithelium.

Light micrograph photos during culture and before application show a scattering of fibroblasts in and around the foam structure (Figure 4A). Following keratinocyte application to the fibroblast plasma-gel matrix, light microscopy demonstrated the development of keratinocyte sheets in areas. This was confirmed by immunohistochemical staining for cytokeratin on the spare CCS (Figure 4B). Immunofluorescence was performed and showed numerous keratinocytes (stained positive with BovK—green) and counterstained fibroblast nuclei (with propidium iodide—red; Figure 4C). The majority of cells had settled in the deeper section of the composite as revealed by confocal microscopy. Cell detachment from the foam edge, and clumping, was evident from the stained composite (Figure 4D), possibly because
of lifting and stretching of the CCS from the culture vessel before application. During histological analysis of the spare CCS, vertical cutting (from superficial to deep) through the foam proved difficult. In an attempt to confirm the presence of a stratified epithelium on the CCS, horizontal sectioning was performed (cuts parallel to the superficial surface).

Cultured Composite Skin Alone (No BTM)
Applying a 1-mm thick CCS directly to a wound bed (ie, without any BTM) revealed that the composite is capable of two different actions: 1) Acting as a delivery vehicle with cells being deposited from the composite, which enabled cell attachment and development of an epithelium, whereas the foam itself was “shed” from the wound surface (Figure 5). 2) Composite “take” (an integrated skin replacement) 14 days postapplication (day 42) with a neo-epithelium integrated within the foam (Figure 6).

The composite applied directly to the wound bed for pigs 1 to 3 illustrates these different outcomes. Figure 5A through D demonstrates a temporal sequence following composite application into a fresh wound. The CCS alone from pig 1 demonstrated a similar overlying “stiff carapace” phenomenon, as seen in some of the BTM–CCS wounds. Clinically by day 10, the majority of the wound (Figure 5E) displayed a dark staining (because of silver deposits from the Acticoat dressing) that, when lifted (Figure 5F), displayed a matt, robust epithelium.

Figure 4. Representative micrographs of the CCS. A, Spindle-shaped fibroblasts in the polymer foam scaffold. B, A continuous sheet (monolayer) of keratinocytes, detached from the polymer matrix, staining immunopositively for cytokeratin, with a central immunonegative area containing fibroblasts with elongated nuclei. Keratinocytes at higher power (inset). C, Immunostaining of keratin with BovK, green and fibroblast nuclei stained red with propidium iodide. D, Stained as per C but higher power showing detachment of cells and clumping of keratin-positive cells (green).

Figure 5. A temporal series of clinical and histological appearance of the cultured composite skin applied alone to pig 1, showing the composite as a delivery vehicle for cells. A, Day 3. B, Day 7. C, Day 10. D, Day 14. E, At day 10, the CCS appears as an overlying “stiff carapace” and when lifted. F, An epithelium is present below. G, Higher power of the reepithelialization extending to the center of the wound. H, H&E section confirming a delivery vehicle, as no polymer matrix visible. I, Positively stained (brown) keratinized epithelium with BovK. J, PAS staining showing basement membrane formation day 10 postapplication. Ks, keratinocytes; BKs, basal keratinocytes; Fbs, fibroblasts/collagen.

(Figure 5G). On histological analysis, sections from pig 1 had no polymer matrix visible, but a well-developed keratinized epidermis had formed, closing the wound (Figure 5H and I). A well-defined basement membrane was demonstrable by day 7 (Figure 5J). This CCS had thus acted as a “delivery vehicle” for cells. This was in contradistinction to pig 2, which displayed composite skin “take” (Figure 6A through D). Clinically, the cultured composite from pig 2 completely integrated by day 7 with no polymer structure visible and exuberant vascularity (Figure 6B) and 70% clinically reepithelialized by day
10 (Figure 6C). This wound was supple to handle and appeared almost completely healed (96%) with a flaking keratin surface by day 14 postapplication (Figure 6D), again with some silver staining. No matrix “shedding” was seen in this wound. Clinically, central reepithelialization was clearly evident on examination at day 10 (Figure 6E and F) and confirmed with a punch biopsy displaying a well-developed epithelium and incorporated 1-mm CCS (Figure 6G). H&E sections from day 14 also confirmed “take” with epithelial integration around the matrix (Figure 6H). Pig 3 similarly demonstrated foam integration; however, reepithelialization was not as prominent as pigs 1 and 2. Pig 3 histology showed plasma cell infiltration on the surface at day 7. By day 14, clinically, 70% of the wound had reepithelialized. A further 20% was covered by a polymer crust, which exhibited underlying epithelium. The CCS here had clinically acted as a delivery vehicle for cells rather than demonstrating composite “take.”

At day 14 postapplication, the mean evaporative water readings for pigs 1 to 3 was 29.9 g/m² for the composites. Skin graft readings were comparable to the composites alone (no BTM) at day 14 post-CCS application, with a mean reading of 20.4 g/m², indicative of stratum corneum formation. The evaporative water loss for the BTM–CCS wounds showed a steep decline from day 7 postapplication (241 to 72.9 g/m²). Although the results suggest a trend of high evaporative water loss within the first week of composite application, with a subsequent fall in evaporative water loss in the following week, the small numbers involved preclude meaningful statistical analysis.

**DISCUSSION**

The BTM has been designed as the first of a two-stage strategy to replace the skin autograft. Literature reports documenting the general failure of keratinocyte application alone in closing integrated dermal matrices are common, and it was foreseen that the second stage of our strategy would be a cultured “composite” skin with dermal and epidermal elements.

Development of the cultured skin substitute has been the next step in generating an alternative to split-thickness skin grafts and CEAs alone. They have been shown to assist and reduce donor skin harvesting with large burns although limitations still exist. Our composite, like many other substitutes on the market or under investigation, currently lacks vascular elements and is devoid of pigment cells. The lack of dermal microvasculature may have contributed to graft loss. However, on delamination and debride- ment of the superficial surface, the wound bed had been temporized by the integrated BTM and displayed excellent vasculature, thus reducing the need for a completely vascularized CCS in the future. The difficulties with the BTM seal and early delamination also produced wound beds with variable thicknesses of granulation tissue. The type of wound bed to which the composite was applied may have affected “take.” The location of cells within the CCS (ie, whether they were superficial, intermediate, or deep within the foam) may also have contributed to the different degrees of CCS integration.

Generally CEAs and many substitutes, whether for clinical application or in vitro investigations, have a culture period of >3 weeks. Although the CCS
was transplanted on day 28, this was merely because of the unavailability of the premade sterilized dishes. The CCS would have otherwise been available for grafting by day 21. Lower cell seeding densities were inoculated in the 1-mm polymer matrix (4 × 10^4/cm^2 for fibroblasts and 2.5 × 10^5/cm^2 for keratinocytes) when compared to higher cell densities used by others. Although inoculation at these cell densities did not produce an identifiable continuous stratified epidermis before transplantation of the CCS, small central areas of developed epidermis were evident from H&E staining of punch biopsies on day 3. This indicates the presence of an exogenous source of keratinocytes, which appeared to accelerate the reepithelialization process and provide wound closure to some of the wounds. From previous studies and from the prestudy, where BTM was used as a control, edge epithelialization was only evident ~1 cm from the wound edge at 18 days postapplication. The dermabrasion pre-CCS application was designed to ensure not only that the BTM surface was refreshed, but also that any edge epithelialization was removed. This would suggest that the source of the cells generating central epithelialization derived from the CCS, either as a delivery vehicle or during CCS “take.” Additionally, the change of dressings at day 3 may have been deleterious. Other users of CEA and/or substitutes generally leave the wound untouched for 5 to 7 days with minimal trauma to the wound area, and this will be assessed for future studies.

In the CCS study, despite the issues generated by the BTM seal and CCS contamination in three composites, the results overall are favorable. The CCS was successful in integrating into a “naked” wound and generating dermal elements and a keratinized, stratified squamous epithelium by day 14, anchored together by a basement membrane well developed by day 7. In other wounds, specifically over an integrated BTM, the CCS acted either as a cellular delivery vehicle (again allowing generation of a bilayer repair with keratinized, stratified squamous epithelium with basement membrane, with the scaffold being lost from the surface) or as a true, integrating cultured bilayer, where foam elements are maintained within the resultant repair.

CONCLUSIONS

A CCS was produced, containing both fibroblasts and keratinocytes, allowing the complex intercellular relationships to begin before clinical application. This was confirmed, providing proof of concept of the designed two-stage strategy (using a sealed BTM to temporize the wound, simultaneously creating a composite, which is then applied to close the wound). The effect of the CCS in “naked” wounds may be important clinically, potentially being applicable for reconstructive surgery or possibly useful in chronic wound management.

These results close out the initial development program. However, further optimization studies are required to generate a CCS with a stratified epithelium before application; this would provide earlier wound closure and thus further reduce wound contraction.

CONFLICT OF INTEREST

The NovoSorb™ biodegradable polyurethane platform is produced by PolyNovo Biomaterials Pty Ltd of Port Melbourne, Victoria, Australia. A joint venture company, NovoSkin Pty Ltd has been established to explore the commercial applications of NovoSorb™ in deep burn injury. Professor Greenwood has a 20% ownership share of NovoSkin Pty Ltd.

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