Development of a Three-Dimensional Human Skin Equivalent Wound Model for Investigating Novel Wound Healing Therapies

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Numerous difficulties are associated with the conduct of preclinical studies related to skin and wound repair. Use of small animal models such as rodents is not optimal because of their physiological differences to human skin and mode of wound healing. Although pigs have previously been used because of their human-like mode of healing, the expense and logistics related to their use also renders them suboptimal. In view of this, alternatives are urgently required to advance the field. The experiments reported herein were aimed at developing and validating a simple, reproducible, three-dimensional ex vivo de-epidermised dermis human skin equivalent wound model for the preclinical evaluation of novel wound therapies. Having established that the human skin equivalent wound model does in fact “heal,” we tested the effect of two novel wound healing therapies. We also examined the utility of the model for studies exploring the mechanisms underpinning these therapies. Taken together the data demonstrate that these new models will have wide-spread application for the generation of fundamental new information on wound healing processes and also hold potential in facilitating preclinical optimization of dosage, duration of therapies, and treatment strategies prior to clinical trials.

Introduction

Skin, the largest organ in the body, performs many important roles, such as barrier protection from physical or chemical insults, sensory functions, and regulation of homeostasis. However, damaged skin cannot perform these critical functions and numerous complications such as infection or fluid loss can occur. Our current understanding of the biological processes underlying wound repair, along with maintenance of skin function and integrity, is limited. This lack of knowledge has arisen in part from the less than ideal in vitro and ex vivo models available for fundamental skin research. The development of improved models is therefore critical to advance our understanding of human skin repair and regeneration. In particular, improved models are required to facilitate the development and preclinical evaluation of wound therapies. Wounds represent a significant and increasing burden on healthcare systems globally and new therapies and wound management approaches are urgently required. The experiments reported herein were therefore aimed at critically evaluating and refining a three-dimensional (3D) ex vivo de-epidermised dermis (DED) human skin equivalent (HSE) model for preclinical evaluation of wound therapies.

Several HSE models have previously been developed with various dermal substrates, including DED, allogerm, collagen matrices with or without glycosaminoglycans and fibroblasts, as well as scaffolds of benzyl ester of semi-synthetic hyaluronan seeded with fibroblasts. The model that we utilized, the DED-HSE, is often considered more physiologically relevant as it incorporates a human acellular dermis to create a multilayered epidermis and retains the basement membrane. This has been previously shown to be critical for keratinocyte attachment in vitro.

Several approaches have been used to create wounds and for studying wound responses in excised skin and/or in vivo models, including scratch tests, tape abrasion, burns, thermal injury, suction blisters, use of electrokeratome/dermatomes, scalpels, meshers, biopsy punches, liquid nitrogen, and lasers. However, a reproducible wound with a given size and depth in an ex vivo DED-HSE model has yet to be reported. The study reported here therefore focused on the development of simple and reproducible models of wound healing using biopsy punches to

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create wounds in the DED-HSEs. To validate the wound model we tested the effect of two novel wound healing therapies—a vitronectin:growth factor (VN:GF) complex currently in clinical trials and a synthetic biomimetic gel.

**Methods**

Approval was obtained from the Queensland University of Technology Review Board and written informed consent was obtained from patients undergoing elective abdominal and breast reduction surgeries before skin collection. The study was conducted with strict adherence to the Declaration of Helsinki Principles.

**Cell isolation and culture**

Keratinocytes were isolated from the skin collected from surgical discards and cultured in Full Green’s (FG) medium following previous protocols. For fibroblast isolation, dermal pieces were finely minced (0.2 cm) and immersed in 0.05% collagenase A (type I; Invitrogen, Mulgrave, Australia) solution in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) at 37°C, 5% CO₂ for 18 h. The enzyme solution was then centrifuged at 2000 rpm for 10 min. Cells were seeded into T80 flasks in 10% fetal bovine serum in DMEM and cultured at 37°C, 5% CO₂.

**Construction of DED-HSE**

The decellularized DED was prepared as described previously with modifications. Sterile stainless-steel rings with an internal diameter of 9 mm were placed onto the papillary side of each DED piece (1.8 cm × 1.8 cm). Keratinocytes (passage 1, 3.6 × 10⁴) were transferred into the ring placed on the DEDs and incubated for 2 days. The rings were removed and the DED-HSEs were then lifted to the air:liquid interface using a stainless-steel grid in approximately 6 mL of FG medium.

**2D multiphoton microscopy images**

The DED-HSEs prepared as described above were cultured in FG medium for 4 days at the air:liquid interface at 37°C, 5% CO₂. Samples were then scanned using a Multiphoton Microscope (JenLab GmbH, Jena, Germany) and cellular autofluorescence was recorded every 2 μm at depths from 0 to 150 μm.

**Use of the DED-HSE model for function-blocking antibody studies**

The DED-HSEs were prepared as described earlier. At the time of being lifted to the air:liquid interface, the DED-HSEs were treated with topical applications of 40 μL VN:GF (0.6 μg VN + 0.6 μg insulin-like growth factor [IGF] binding protein 3 [IGFBP3] + 0.2 μg IGF + 0.2 μg epidermal growth factor [EGF]) (Tissue Therapies, Brisbane, Australia); 36 μL of VN:GF (0.54 μg VN + 0.54 μg IGFBP3 + 0.18 μg IGF + 0.18 μg EGF) + anti-IGF-1 receptor antibody (anti-IGF-1R) (1:10; Calbiochem, Darmstadt, Germany); or 40 μL of VN:GF (0.6 μg VN + 0.6 μg IGFBP3 + 0.2 μg IGF + 0.2 μg EGF) + nonspecific IgG1 subtype (IgG) (1:500; Chemicon, Temecula, CA). After 7 days of culture at the air:liquid interface, the DED-HSEs were stained with methylthiazol tetrazolium (MTT) and fixed for histological analysis.

**Fabrication of the partial-thickness DED-HSE wound model and assessment of the VN:GF and the synthetic biomimetic gel**

After 9 days culture at the air:liquid interface, 6 mm partial-thickness excisional wounds were created in the DED-HSEs with a 6 mm biopsy punch (Stiefel, Castle Hill, Australia) excising through the epidermis. The epidermis was then peeled away from the DED with forceps and discarded. To assess the effect of VN:GF in wound healing, wounded areas of the DED-HSEs were cultured with 6 mL of FG, VN:GF (0.23 μg/mL VN, 0.23 μg/mL IGFBP3, 0.08 μg/mL IGF and 0.08 μg/mL EGF), or serum-free media (SFM) media at the air:liquid interface for 3, 7, and 12 days. To evaluate the effect of the synthetic biomimetic gel, the wounded areas of the DED-HSEs were treated with either topical application of 13 μL of gel (2.25%, w/v) or 13 μL of FG (positive control) for 7 days. Samples were then stained with MTT, hematoxylin and eosin, or immunohistochemistry and analyzed microscopically as described previously.

**Fabrication of the full-thickness DED-HSE wound model and assessment of the synthetic biomimetic gel**

Sterile stainless-steel rings with an internal diameter of 6.7 mm were placed on the papillary (for seeding of keratinocytes) or reticular (for seeding of fibroblasts) face of the DEDs. Keratinocytes and fibroblasts (2.0 × 10⁶ per ring) were seeded into the center of the rings. The DED-HSEs were sealed with cells and incubated at 37°C, 5% CO₂ as follows: for DED-HSEs seeded with keratinocytes alone, cells were seeded for 48 h; for DED-HSEs seeded with fibroblasts alone, cells were seeded for 72 h; for DED-HSEs seeded with keratinocytes and fibroblasts together, fibroblasts were seeded for 72 h and keratinocytes were then seeded on top of fibroblasts for a further 48 h. The DED-HSEs were then lifted to the air:liquid interface. After 7 days, 4 mm diameter full-thickness incisional wounds were created in the DED-HSEs using a 4 mm biopsy punch (Stiefel), which cut through all the layers of the DED-HSEs, both epidermal and dermal. This was followed by removal of the 4 mm diameter epidermal/dermal core. The wounds were injected with 13 μL of synthetic biomimetic gel (2.25%, w/v) or collagen gel. The wounded DED-HSEs were probed using histology and immunofluorescence after 14 days.

**Formation of synthetic biomimetic gel and the collagen-I hydrogel**

Synthetic biomimetic gels were produced as previously described. Briefly, peptides containing complementary substrates for FXIII-catalyzed crosslinking, NQEQQVS PLERCG-NH₂ (TG-Gln) or Ac-FKGGGPQOG-IWGQERCG-NH₂ (TG-MMP-Lys; NeoMP, Strasbourg, France), were grafted onto eight-arm poly(ethylene glycol) macromolecules (8-PEG, Mᵡ = 40 kDa) yielding the hydrogel precursors 8-PEG-Gln and 8-PEG-MMP-Lys, respectively. After the coupling reactions the solutions were dialyzed against ddH₂O and were subsequently freeze-dried. The TG-MMP-Lys peptide
also includes a matrix metalloproteinase (MMP) substrate (underlined; † indicates cleavage position) to render the final hydrogels susceptible to proteolytic degradation. For synthetic biomimetic gels insensitive to MMP degradation, the 8-PEG-MMP-Lys precursor was replaced by 8-PEG-noMMP-Lys in which the MMP-degradable substrate was substituted by the MMP-insensitive sequence -GDQGIA GF- (NeoMP).

Gels were subsequently formed by FXII-catalyzed crosslinking of stoichiometrically balanced 8-PEG-Gln and 8-PEG-MMP-Lys (for MMP-sensitive gels) or 8-PEG-noMMP-Lys (for MMP-insensitive gels) in Tris buffer (TBS, 50 mM, pH 7.6) containing 50 mM calcium chloride and 10.7 U/mL activated FXIII. For example, to produce 120 μL of 5% (w/v) hydrogel matrices the following solutions were used: 43.17 μL (8-PEG-Gln, 67.64 mg/mL), 45.83 μL (8-PEG-MMP-Lys or 8-noMMP-Lys, 67.20 mg/mL), 19 μL spare volume (for the incorporation of RGD-peptide or Tris buffer for blank gels), 6 μL calcium chloride (1 M), and 6 μL of activated FXIII (213.5 U/mL; kindly donated by Baxter, Vienna, Austria). For hydrogels with 1.5% and 2.25% (w/v) dry mass, the precursor solutions were diluted accordingly. RGD-functionalized hydrogels (50 μM) were produced by addition of the peptides NQEQLVSL-GRGDSPG-NH₂ (TG-Gln-RGD). Crosslinking reactions were performed at 37°C and 5% CO₂ humidified atmosphere for 30–40 min.

Collagen gels were produced from a single batch of rat tail collagen type I solution (BD Biosciences, North Ryde, Australia). Specifically, to produce a 100 μL volume of gel, 89.6 μL of collagen-I stock solution (3.35 mg/mL), 2.42 μL sodium hydroxide (1 M), and 8 μL of 10×phosphate-buffered saline (PBS; Oxoid, Victoria, Australia) were used. Collagen-I hydrogels were formed at 37°C, in a 5% CO₂ atmosphere for 60 min.

Immunofluorescence

The full-thickness wounded DED-HSEs injected with synthetic biomimetic gels were probed for nuclei (4′,6-diamidino-2-phenylindole [DAPI], 1:2000; Vector Laboratories, Burlingame, CA), F-actin (phalloidin rhodamine) (1:250; Molecular Probes, Eugene, OR), and pan-cytokeratin antibodies (Biolegend, San Diego, CA, and Bioimage Systems, Wetzlar, Germany) at λex = 356 nm, λem = 461 nm for DAPI/nuclei visualization; λex = 557 nm, λem = 574 nm for phalloidin rhodamine/actin filament visualization; and λex = 495 nm, λem = 515 nm for fluorescein isothiocyanate/pancytokeratin visualization.

Statistics

Means ± standard error of the mean are presented. Duplicate DED-HSEs were tested individually with each treatment within each assay, and the entire experiment was repeated three times. The DEDs and cells for each replicate experiment were isolated from three different donors. The data were pooled from three replicate experiments in which each treatment was tested in duplicate. One-way analysis of variance with Tukey’s post hoc tests (all group comparisons) were used to analyze the data. Statistically significant differences were determined as p < 0.05.

Results

Similarity between native human skin and the DED-HSE

To compare the DED-HSE constructed as described in the methods with native human skin, samples were first investigated using multiphoton microscopy. The multiphoton microscopy enables the nondestructive examination of cellular changes, such as morphology, in real-time. For the DED-HSE, as seen in Figure 1a, the stratum corneum appeared bright with strong contrast because of a high concentration of the endogenous fluorophore keratin. Fifty micrometers below the surface, the stratum spinosum was visualized and exhibited dense cell formation and decreasing cell diameter. Keratinocytes could be clearly separated (Fig. 1b). At depths of 100–150 μm below the surface, the innermost epidermal cell layer (stratum basale) was found (Fig. 1c). Basal keratinocytes appeared as small and polygonal cells with dark nuclei. The dark and oval areas, which correspond to the connective tissue of the papillary dermis, were observed interrupting the stratum basale. For the native skin, the captured images showed similar cellular morphology and organization to the DED-HSE (Fig. 1d–f). These data indicate that morphology and organization of keratinocytes between ex vivo skin and the DED-HSE were comparable.

Development and validation of a 6 mm diameter partial-thickness DED-HSE wound model

To determine an appropriate protocol for creating a reproducible partial-thickness wound, the DED-HSEs were cultured at the air:liquid interface for 7, 9, and 11 days, and the epidermis was peeled from each DED-HSE after excision with a 6 mm biopsy punch (Fig. 2a). We found that the epidermis of DED-HSEs cultured for 7 days would not separate from the dermis (Fig. 2b) with many keratinocytes remaining attached to the dermis in the area of excision. However, after 9 days the epidermis could be removed from the dermis. Although the epidermis could be peeled away as a whole piece from the dermis after 11 days, the majority of cells at the edge of the defect displayed a differentiated phenotype, as determined by histology.

To establish that the wounded DED-HSEs were capable of healing, the constructs were cultured in serum-containing FG medium for 3, 7, and 12 days, respectively. The uncovered wound area, as revealed by MTT assay, decreased from 26.49 ± 1.18 mm² at day 0 to 19.11 ± 3.44 mm² at day 3, to 11.25 ± 2.58 mm² at day 7, and to 7.53 ± 2.92 mm² at day 12 (Fig. 2c, d).
Histological analysis allowed evaluation of the wounded DED-HSEs throughout the healing process (Fig. 2c, e). After 3 days the wound bed of the DED-HSEs was partially covered by a wedge-shaped “epithelial tongue,” two to three cells in depth. After 7 days, a monolayer or bilayer of keratinocytes covered the center and a more stratified epithelium was evident toward the wound margins. A stratified epithelium covered the entire area at day 12. The lateral migration of keratinocytes (distance from two of the wound edges to the center of the wound in the DED-HSEs) increased from zero at day 0, to 1.19 ± 0.51 mm after 3 days, to 3.10 ± 0.63 mm after 7 days, and to 4.18 ± 0.77 mm after 12 days. Taken together, these data indicate that the wound in the DED-HSE is capable of “healing.”

Immunohistochemistry was undertaken to detect the expression of specific markers on the surface of cells in the wounded DED-HSEs (Fig. 2f). Immunohistochemical analysis with a monoclonal antibody raised against the nuclear transcription factor p63 revealed the presence of undifferentiated proliferating cells. At day 3, intense immunoreactivity of p63 was detected in keratinocytes located at the edge of the wound within the suprabasal and basal layers of the wounded DED-HSEs. However, the immunoreactivity was decreased at day 7 and limited to the deeper basal layers at day 12. Immunohistochemical analysis with a monoclonal antibody raised against keratins 1, 10, and 11 revealed the presence of differentiated cells. The positive immunoreactivity of keratins 1, 10, and 11 was present at the edge of the wounded DED-HSEs at days 3, 7, and 12.

FIG. 1. Multiphoton microscopy images of the de-epidermised dermis human skin equivalent (DED-HSE) (a–c) and the native human skin (d–f). Skin samples were scanned using multiphoton microscopy. Images captured at different tissue depths reveal the stratum corneum (a, d), the stratum spinosum (b, e), and the basal layer (c, f). Autofluorescence was recorded in 2 μm increments, up to 150 μm below the surface. Scale bar: 300 μm.

FIG. 2. Development and validation of a 6 mm diameter superficial-thickness DED-HSE wound model. (a) Photograph of technique used to create the superficial-thickness wound in the DED-HSE. A wound was created using a 6 mm biopsy punch, followed by the removal of a central portion of the epidermis with forceps. (b) Methylthiazol tetrazolium (MTT) (left panels) and histological analysis (right panels). A 6 mm superficial-thickness wound was created in individual DED-HSE after culture at the air:liquid interface for 7, 9, and 11 days. Dashed lines indicate the wound boundaries. (c) Representative images of MTT and hematoxylin and eosin-stained DED-HSEs during repair. Samples were cultured with Full Green’s (FG) medium for 3, 7, and 12 days after superficial-thickness wounds were created. Dashed lines indicate the wound boundaries. (d) Quantification of the uncovered wound area and (e) the lateral migration of keratinocytes. (f) Representative images of the p63 and keratins 1, 10, and 11 (K1/10/11) expression in DED-HSEs during repair. n = 6; *p < 0.05. Scale bars: 6 mm (a), 0.6 mm (b), 3 mm (c, top), 300 μm (c, bottom), and 100 μm (f). Color images available online at www.liebertonline.com/ten.
FIG. 3. Investigation of the effect of vitronectin-growth factor (VN:GF [VG]) on healing of superficial-thickness wounds. Representative images of (a) MTT and (b) histological analysis of the wounded DED-HSEs. Samples were cultured with FG, VN:GF, and serum-free media (SFM) for 3, 7, and 12 days after the creation of 6 mm superficial-thickness wounds. Dashed lines indicate the wound boundaries. Quantification of (c) uncovered wound area and (d) lateral migration of keratinocytes. 
(e, f) Mechanistic investigation using the DED-HSEs. Samples were topically treated with VN:GF, VN:GF + IGF-1R Ab, and VN:GF + IgG (nonspecific IgG1 subtype) for 7 days. $n = 6$; *$p < 0.05$. Scale bars: 3 mm (a), 300 µm (b), and 50 µm (e). Color images available online at www.liebertonline.com/ten.
Investigation of the effect of VN:GF on partial-thickness wound healing

Wounded DED-HSEs were cultured with serum-containing FG, VN:GF, or SFM and the area of the exposed DED was measured. The uncovered wound area cultured with FG and VN:GF treatments reduced from 26.49 ± 1.18 mm² at day 0 to 15.91 ± 4.29 and 18.54 ± 3.75 mm² at day 3, to 10.9 ± 4.07 and 9.56 ± 2.17 mm² at day 7, and to 7.64 ± 3.51 and 6.44 ± 1.70 mm² at day 12, respectively, as revealed by MTT assay (Fig. 3a, c). These areas were significantly smaller (p < 0.05) than those observed in DED-HSEs treated with SFM at days 7 and 12.

The wounded DED-HSEs were also investigated through histological analysis (Fig. 3b, d). The wound beds of DED-HSEs cultured with either FG or VN:GF treatments were partially covered by a wedge-shaped “epithelial tongue,” two to three cells in thickness, after day 3; a monolayer or bilayer of keratinocytes in the center and a more stratified epithelium toward the wound margins were evident at day 7. A fully stratified epithelium was present after 12 days. In contrast, DED-HSEs cultured with SFM exhibited only a few keratinocytes encroaching from the edge of the wound after day 3 and these disappeared by day 7. The lateral migration of keratinocytes cultured with FG and VN:GF treatments increased from zero at day 0 to 1.73 ± 0.54 and 2.10 ± 0.92 mm at day 3, to 3.49 ± 0.92 and 3.44 ± 0.35 mm at day 7, and to 4.12 ± 0.99 and 4.67 ± 0.47 mm at day 12, respectively. Again, these distances were significantly greater (p < 0.05) than those observed on DED-HSEs cultured with SFM (0 ± 0 mm). Taken together, these data indicate that the wound area in DED-HSEs cultured with FG and VN:GF treatments continued to decrease, whereas the wounds in DED-HSEs cultured with SFM remained uncovered.

To validate the potential of the model for studies exploring the mechanisms underpinning the VN:GF-complex-stimulated migration, proliferation, and differentiation of keratinocytes, the DED-HSEs were treated daily with VN:GF, VN:GF + IGF-1R Ab (anti-IGF-1 receptor antibody), and VN:GF + IgG (nonspecific IgG1 subtype) for 7 days. These treatments have been examined in 2D cell monolayer previously by our laboratory and demonstrated that the enhanced cellular functions stimulated by the VN:GF complex involve the IGF receptor (IGF-1R). In the 3D DED-HSE studies reported herein, the thickness of the epidermis in the DED-HSEs exposed to these three treatments were 170.42 ± 2.9, 80.99 ± 2.32, and 161.45 ± 4.37 μm, respectively (Fig. 3e, f). This indicates that the IGF-1R Ab can significantly block the effect of VN:GF on epidermal formation and also demonstrates that the 3D DED-HSE model has utility for mechanistic studies.

Investigation of the effect of a synthetic biomimetic gel on partial-thickness wounds

The partial-thickness wounded DED-HSE model was also utilized to assess the effect of a synthetic biomimetic gel. Hydrogel with RGD peptides (the synthetic biomimetic gel), hydrogel without RGD peptides, and serum-containing FG medium were topically applied to the wounds and were assessed along with controls with no treatment (Fig. 4). The area of the wound remaining uncovered with keratinocytes in DED-HSEs were 2.13 ± 1.05 mm² (hydrogel with RGD peptides), 3.64 ± 1.14 mm² (hydrogel without RGD peptides), 8.33 ± 2.22 mm² (positive control FG medium), and 10.70 ± 1.50 mm² (no treatment), respectively (Fig. 4a, b).

The morphology of keratinocytes in the wounded DED-HSEs was examined through histological analysis (Fig. 4a). After day 7 the wound bed of DED-HSEs treated with hydrogel containing RGD peptides was almost completely covered with a stratified epithelium. On the other hand, wounded areas of DED-HSEs treated with the positive control FG medium, or no treatment, were only partially covered by multilayers of keratinocytes in the center and a more stratified epithelium toward the wound margins. After 7 days the lateral migration of keratinocytes in wounds treated with hydrogel with RGD peptides, hydrogel without RGD peptides, FG medium, and no treatment were 4.66 ± 0.54, 4.42 ± 0.62, 3.73 ± 1.01, and 2.46 ± 0.21 mm, respectively (Fig. 4c). These results indicate that the synthetic biomimetic gel can significantly enhance keratinocyte reepithelialization.

Development of a full-thickness DED-HSE wound model (seeded with keratinocytes and fibroblasts alone and together) and investigation of the synthetic biomimetic gel in full-thickness wound healing

To investigate the effect of the synthetic biomimetic gel in full-thickness wound healing, full-thickness wounds were created using a 4 mm biopsy punch excising through the full depth of the DED-HSE and removing the epidermal/dermal core (Fig. 5a). The excised wound in the DED-HSEs were then filled with the synthetic biomimetic gel and analyzed with immunofluorescence. When DED-HSEs seeded with keratinocytes alone were wounded, no cells were detected in the wound after 14 days (Fig. 5b). Conversely, when DED-HSEs seeded with fibroblasts alone were wounded, a large number of fibroblasts were found to have migrated into the wound (Fig. 5c). For the wounded DED-HSEs produced with keratinocytes and fibroblasts cocultured together, keratinocytes migrated across the wound as detected by pan-cytokeratin expression (Fig. 5d).

The morphology of the wounded DED-HSEs was also investigated through histological staining. When DED-HSEs seeded with fibroblasts alone were wounded, the fibroblasts had migrated into the synthetic biomimetic gel (Fig. 5f). Curiously, when DED-HSEs seeded with keratinocytes alone were wounded, the keratinocytes migrated along the dermis at the edge of the wound rather than migrating across the wound (Fig. 5e). With DED-HSEs that had been cocultured with keratinocytes and fibroblasts together and were then wounded, histological analysis confirmed that the keratinocytes migrated into the wound area (Fig. 5g). Taken together, these results indicate that the synthetic biomimetic gel was able to support the migration and proliferation of fibroblasts, as well as keratinocytes, albeit only in the presence of fibroblasts.

Further investigation of the cellular responses to the synthetic biomimetic gel

To further characterize the synthetic biomimetic gel, full-thickness wounds in the DED-HSEs seeded with fibroblasts were filled with hydrogels with/without MMP cleavage sites and RGD peptides. Immunofluorescent analysis revealed significant differences in cellular responses to hydrogels with and without incorporated MMP cleavage sites or RGD
peptides (Fig. 6). When wounded DED-HSEs were treated with collagen gel or hydrogel incorporating the MMP cleavage sites and RGD peptides (the synthetic biomimetic gel), large numbers of fibroblasts were found migrating within the hydrogel in the wound area. Conversely, when the wounded DED-HSEs were left untreated, or treated with hydrogel without MMP cleavage sites or without RGD peptides (Gel − RGD), few cells were observed to migrate into the gel filling the wounds. These data indicate that the RGD peptides and MMP cleavage sites have a significant influence on the migration and proliferation of fibroblasts into the synthetic biomimetic gel.

Discussion

Many challenges are associated with conducting relevant fundamental and preclinical studies of human skin maintenance and wound repair; however, HSE models are emerging as promising candidates. In particular, HSEs incorporating DED appear to be superior, predominantly because of their anatomical, structural, and functional similarities to human skin. The study reported herein provides further confirmation of the similarities between native human skin and DED-HSEs using a novel technique, multiphoton microscopy. Thus we showed that the DED-HSEs have cellular morphology and organization consistent with native human skin (Fig. 1).

To efficiently evaluate the efficacy of novel wound healing therapies, a reliable and reproducible 3D human skin wound model that more closely represents the in vivo environment, as well as contains cells and proteins that are human in origin, is clearly required. This study was therefore directed at developing a reproducible partial-thickness DED-HSE wound model. The partial-thickness DED-HSE wound model...

FIG. 4. Investigation of the effect of a synthetic biomimetic gel on repair of superficial-thickness wounds. Synthetic biomimetic gels were injected into 6 mm superficial-thickness wounds created in the DED-HSEs and cultured at the air-liquid interface for 7 days. (a) Representative images of MTT and (b) hematoxylin and eosin-stained DED-HSEs during repair. Superficial-thickness wounds in DED-HSEs were topically treated with gel with RGD peptides (Gel + RGD), gel without RGD peptides (Gel − RGD), FG medium, and no treatment (NT) for 7 days. The dashed lines indicate the initial boundary of the wounds. Quantification of the uncovered wound area (b) and lateral migration (c) of keratinocytes from the wound edge to the center of the wound. n = 6; *p < 0.05. Scale bars: 3 mm (a, top) and 300 μm (a, bottom). Color images available online at www.liebertonline.com/ten.
model reported herein, created by excising a central portion of the epidermis through use of a 6 mm biopsy punch, demonstrated the ability to “heal,” as revealed by MTT and histology. Interestingly, the repaired epithelial layer formed had a similar morphology and keratin marker expression profile to that reported in other studies in humans.27,40

Odland and Ross have previously reported the creation of partial-thickness skin incisions in humans to study wound repair. They demonstrated that the cells at edges of the incision started to migrate as an “epithelial tongue” within 48 h after wounding. The reepithelialization was reported to be complete within 72 h and a well-stratified epithelium was formed by 96 h after wounding.40 This is somewhat faster than the healing response we observed in the DED-HSEs. However, this difference is most likely due to the fact that Odland and Ross used an incisional wound in humans, and our studies used excisional wounds in DED-HSEs. All the same, it may well be that the healing process in the DED-HSEs is slower than that observed in normal human skin in vivo. This may arise from the fact that the DED-HSE ex vivo model does not contain inflammatory cells and a blood supply. Nevertheless, it is clear that the reepithelialization data reported with this partial-thickness wound model are consistent and demonstrate that this model can be used to study wound responses in a way that mimics to some extent in vivo wound healing.

Having established that the DED-HSEs can “heal,” the effect of a novel VN:GF complex in facilitating healing of the excisional wounds was evaluated. Of note, the VN:GF complexes have previously been found to significantly increase reepithelialization in a porcine deep dermal partial-thickness burn model.38 Analysis of the partial-thickness wounded DED-HSE models treated with the VN:GF using MTT and histological analysis revealed enhanced cell migration, proliferation, and differentiation of keratinocytes at
the edges of the wounds (Fig. 3). These results are similar to those observed in wounded DED-HSEs treated with serum-containing FG medium and indicate the potential of VN:GF as, first, a wound-healing therapy, and second, a defined serum-free medium that could potentially replace the widely utilized FG medium. Indeed, our laboratory recently reported that primary keratinocytes can be successfully isolated and cultured to reform an epidermal layer using a fully defined, synthetic serum-free medium containing VN:GF.\textsuperscript{41}

Moll \textit{et al.} and Geer \textit{et al.} have also previously used a biopsy punch to create wounds on human skin and DED-HSEs, respectively.\textsuperscript{23,30} However, Moll \textit{et al.} used a method that required the presence of serum to heal the wounds created in the human skin, whereas Geer \textit{et al.} used a DED-HSE model that required grafting onto a mouse to facilitate healing of the defects. Similarly, another two studies by Bhora \textit{et al.} and Andreadis \textit{et al.} investigated the effects of growth factors such as keratinocyte growth factor, fibroblast growth factor, IGF-1, and EGF\textsuperscript{42,43} on human skin and the DED-HSE wound healing, respectively. However, both of these studies also required the presence of bovine serum albumin or fetal calf serum. Our ability to study the healing of wounds in the absence of poorly defined factors, such as fetal calf serum and/or additives such as pituitary extract, represents a significant advance in that it will facilitate the study of wound healing in a defined environment, as well as have wider utility in the animal-free development and testing of consumer products.

We also describe the successful use of the DED-HSE for mechanistic functional studies via examining the effect of a growth factor treatment (VN:GF) in the presence of a growth factor receptor function-blocking antibody (IGF-1R Ab). This demonstrates that the 3D DED-HSE model has significant utility for 3D mechanistic studies (Fig. 3e, f). This is important, as 2D culture systems provide cells with extremely unnatural geometric, mechanical, and biochemical restrictions.\textsuperscript{44} Consequently, 2D monolayer cell culture systems may result in unsatisfactory, misleading, and nonpredictive data that have minimal relevance to the \textit{in vivo} situation.\textsuperscript{45,46}

The partial-thickness wounded DED-HSE models were also used to test a synthetic biomimetic gel with features designed to be similar to fibrin. During the wound healing process \textit{in vivo}, a fibrin clot forms and provides a primary matrix for cells, assisting them to migrate and permeate the

\textbf{FIG. 6.} Investigation of cell attachment and protease cleavage motifs in the synthetic biomimetic gel on healing of full-thickness wounded DED-HSEs seeded with fibroblasts. Hydrogel incorporating matrix metalloproteinase (MMP) cleavage sites and RGD peptides (Hydrogel + RGD + MMP), hydrogel incorporating MMP cleavage sites without RGD peptides (Hydrogel – RGD + MMP), hydrogel incorporating RGD peptides without MMP cleavage sites (Hydrogel + RGD – MMP), collagen gel, and no gel were injected into full-thickness wounds created in DED-HSEs and cultured at the air:liquid interface for 14 days. Samples were fixed and double-stained with 4′,6-diamidino-2-phenylindole (nuclei, blue) and phalloidin rhodamine (F-actin filament, red). Images were captured with a Leica SP5 Scanning Confocal Microscope. Scale bar: 250 µm.
There are two key features involved in this process: (1) adhesion sites located in the matrix permit cells to attach and migrate into the gel; and (2) the matrix reacts to cell-derived proteolytic activity. This allows cells to degrade the matrix locally by secreting plasmin or MMPs and then to migrate into the matrix without constraint. The incorporation of RGD and MMP cleavage sites into the multifunctional PEG hydrogel described in earlier experiments enables this gel (synthetic biomimetic gel) to imitate two essential biological functions of an extracellular matrix: cell adhesion and protease degradation. A synthetic biomimetic gel mimicking a fibrin clot with attributes that facilitate cell adhesion as well as degradation of the gel by proteases has been developed by Ehrbar et al. They have shown that this synthetic biomimetic gel enhances the migration and proliferation of fibroblasts and enables the formation of an interconnected cellular network. Based on this, we hypothesized that keratinocyte reepithelialization may also be accelerated by this synthetic biomimetic gel. To explore this concept further, synthetic biomimetic gels were injected into the wound areas after the partial-thickness wounds were created in the DED-HSEs. Results from the MTT analysis and histology demonstrated that this gel was able to augment the migration, proliferation, and differentiation of keratinocytes during partial-thickness wound healing (Fig. 4). However, no significant difference was observed in wounded DED-HSEs treated with hydrogel incorporated with RGD peptides (synthetic biomimetic gel) and hydrogel without RGD peptides. Therefore, we believe that the enhanced cellular responses observed in the absence of the synthetic biomimetic gel may be aided by the creation of a liquid-liquid-type interface with the cells at the edges of the wounds. That is, the gels, which were hydrated by the FG medium through absorption, provided moist environments facilitating enhanced migration of the keratinocytes. Indeed, the report by Gilje in 1948 suggested that ulcers provided with a moist environment through an adhesive bandage tape have enhanced healing, which supports our data and the overall concept of “a moist environment to facilitate optimal wound healing.”

To further characterize the synthetic gel, hydrogels with/without MMP cleavage sites and with/without RGD peptides were injected into full-thickness excisional wounds in the DED-HSEs. Consistent with the findings of Ehrbar et al., we observed that with DED-HSEs seeded with dermal fibroblasts, the cells migrated into and proliferated within hydrogel incorporating MMP cleavage sites and RGD peptides (Figs. 5 and 6). The RGD permitted cells to attach and migrate into the gel, whereas the MMP cleavage sites allowed the cells to degrade the matrix locally through the secretion of endogenously produced MMPs. Interestingly, with DED-HSEs seeded with keratinocytes alone, the cells were not detected in the wound area. This may be due to the fact that the synthetic biomimetic gel was rapidly degraded by MMPs secreted by the keratinocytes and hence did not provide the necessary physical scaffold to support keratinocyte migration across the wound. However, when keratinocytes were cocultured with fibroblasts together in the DED-HSEs, the keratinocytes migrated across the wound of the DED-HSE (Fig. 5), albeit this was only observed once. All the same, it has been noted by others previously that fibroblasts play a vital paracrine role in creating a balanced microenvironment for the maintenance and repair of the epidermis and this concept is supported by the data reported here.

Although our results demonstrate the potential of the 3D DED-HSE model for investigations into human skin repair, it is important to bear in mind that the DED-HSEs do not recapitulate all aspects of wound repair, or they do not fully represent a chronic wound. The major drawbacks of current DED-HSEs are the absence of a blood supply, lack of a functioning immune system, and limited wound contraction, albeit human wounds repair primarily via reepithelialization, rather than contraction. We propose that through the addition of additional skin cell types (e.g., melanocytes, Langerhans’ cells, microvascular endothelial cells), many of these criticisms will be partially addressed and will facilitate the creation of a reliable DED-HSE platform technology. The isolation of these cells from skin adjacent to wounds on limbs that have been amputated due to a nonhealing wound may also represent a step forward. We recognize, however, that inclusion of these cells does not create either a circulatory or nervous system as present in vivo. Nevertheless, this approach will facilitate an increased understanding of the interactions between cells that have key roles in skin biology. All the same, it is important to recognize that this lack of vascularization in the HSE models themselves may well be an advantage as the wounds in the HSEs are somewhat hypoxic in comparison to healthy skin, thereby mimicking more closely the hypoxic in vivo chronic wound environment.

In summary, we report here the development of novel approaches to create reproducible partial-thickness and full-thickness wounds with a given size and depth in ex vivo DED-HSEs. Additionally, our studies indicate that the novel VN:GF growth factor combination not only heals excisional wounds in these HSE models, but also provides a defined serum-free ex vivo culture system for the DED-HSEs. Thus these models will facilitate our understanding of human skin repair, regeneration, and maintenance processes and coincidentally provide a relevant in vitro tool for the assessment of potential wound healing therapies in a defined, synthetic environment. This will decrease our reliance on the use of animals for scientific experimentation—an increasingly critical issue, especially given the European Union regulations (76/768/EEC, February 2003) that prohibit the sale of consumer products developed with testing that involves animals. In addition, these models have allowed us to investigate and establish the potential of the VN:GF and synthetic biomimetic gel technologies for the healing of partial- and full-thickness wounds, contributing valuable new information that will further our understanding of the effects of these novel wound healing treatments. This is important for not only generating fundamental new information on wound healing processes, but also for preclinical optimization of dosage, duration of therapies, and treatment strategies, prior to clinical trials. Indeed, studies using these models are likely to be of greater benefit than the numerous wound healing studies that are reported using rodents and other small animals, as few of these translate well to the clinical setting.

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Disclosure Statement

Some of the authors (Z.U., R.D., S.R., and D.I.L.) have a duality of interest that they hold shares in Tissue Therapies Ltd., a biotechnology start-up company that has been spun out of the Queensland University of Technology to commercialize this technology. Z.U. and D.I.L also consult for Tissue Therapies Ltd.

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