

Production of Full-Thickness Human Skin Substitutes Without Animal-Derived Collagen

KR Gratz, BM Steiglit, JC Pirnstill, NJ Simon, SC Pirnstill, SL Sisson, AR Comer, and BL Allen-Hoffmann.
Stratatech Corporation, Madison, WI, USA.

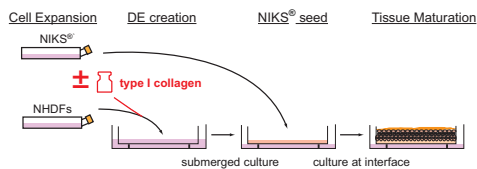
Introduction

Currently-available full-thickness skin substitutes contain a stratified epidermal layer grown on a dermal equivalent (DE) composed of human dermal fibroblasts (NHDFs) in an animal-derived (often bovine) collagen gel. These autopolymerized collagen matrices do not provide the normal composition, structure, or function of the human dermis and despite rigorous screening and quality control, introduce process variability and the potential for adventitious agent transmission (e.g. bovine spongiform encephalopathy). Alternatively, long-term cultures of NHDFs direct the formation of tissues *de novo*, through the secretion and organization of extracellular matrix [1,2].

Objective

Generate NHDF-derived dermal equivalents and use them for production of fully cell-derived mature skin substitute tissues.

Methods



Tissue Production: Fully cell-derived skin substitute tissues (44 cm²) were generated using NHDF-derived DEs and compared to Stratatech's clinically tested StrataGraft[®] skin tissue [3].

Standard collagen DE: NHDFs were mixed with an animal-derived type I collagen solution and polymerized in porous tissue culture inserts.

NHDF-derived DE: NHDFs were seeded directly into tissue culture inserts (5,000 cells/cm²) and maintained in submerged culture for 21 or 28 days to allow *de novo* DE generation.

Skin Substitutes: Proprietary NIKS[®] keratinocyte progenitor cells [4] were seeded onto either standard collagen gel DEs, to form StrataGraft[®] tissues, or 28-day NHDF-derived DEs and cultured at the air-liquid interface to promote epidermal maturation.

Analysis of 21-day and 28-day NHDF-derived DEs:

DE composition and structure

- Macroscopic and histological appearance: H&E
- Thickness: Mitutoyo digital thickness gauge
- Collagen Content: Sirius red assay
- Viability: MTT assay

Analysis of mature skin substitute tissues:

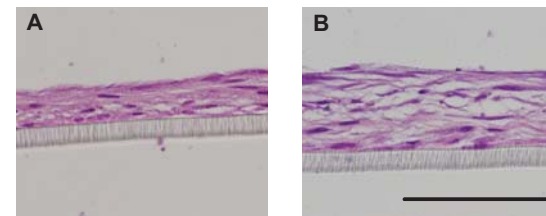
Skin substitute composition and structure

- Histology: H&E, Trichrome, IIF (for elastin)
- Thickness: Mitutoyo digital thickness gauge
- Collagen Content: Sirius red assay
- Viability: MTT assay
- NHDF content: cell counts of tissue digests

Skin substitute function

- Epidermal barrier function: electrical impedance
- Mechanical properties: uniaxial tensile test
- VEGF secretion: ELISA

1. Histological analysis of NHDF-derived DEs



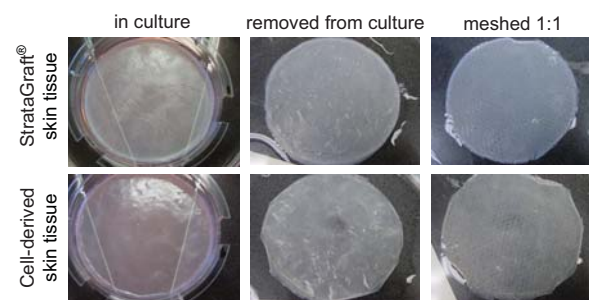
Histology of NHDF-derived DEs after 21 (A) or 28 (B) days in culture. NHDFs were seeded at low density and cultured for either 21 or 28 days. 8mm biopsy punches were taken from several locations across the tissue surface, embedded in paraffin, sectioned, and stained with H&E. A substantial increase in tissue thickness was qualitatively observed over the final week of culture. Bar = 100 µm.

2. Composition and structure of NHDF-derived DEs

DE Properties	Day 21	Day 28
Thickness (µm)	37 ± 3	65 ± 10
Collagen Content (µg/cm ²)	133 ± 7	269 ± 55
Viability - MTT (O.D.)	0.39 ± 0.06	0.47 ± 0.10

Composition and structure of NHDF-derived DEs were analyzed after 21 or 28 days in culture. **Tissue thickness** was averaged from readings at several points across the surface. **Collagen density** was calculated using a colorimetric Sirius red assay (modified from [5]). **DE viability** was determined by MTT assay. For each analysis n = 6 21-day DEs (3 batches); n = 5 28-day DEs (3 batches) were analyzed. Significant **increases** or **decreases** (p<0.05) in 28-day DEs compared to those analyzed at 21 days were determined by t-test.

3. Appearance and handling of skin substitute tissues

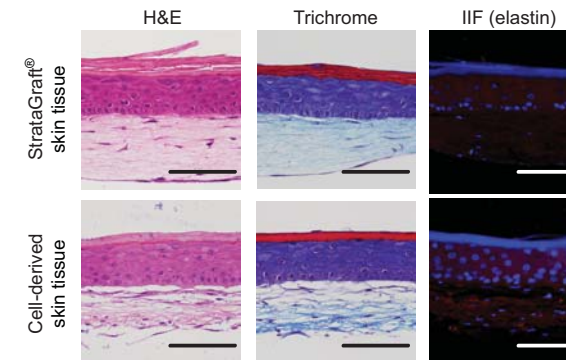


Gross appearance cell-derived skin substitutes was comparable to StrataGraft[®] controls. All skin substitutes demonstrated handling characteristics that would be necessary clinically, including removal from culture inserts and meshing.

Results

4. Histological analysis of skin substitute tissues

Skin substitute tissues were analyzed histologically and compared to StrataGraft[®] controls. Paraffin-embedded sections were stained with H&E or Trichrome to visualize tissue morphology and collagen organization. Both types of tissue displayed fully differentiated epidermal layers, including basal, spinous, granular, and cornified layers. NHDF-derived DEs had a more dense, fibrillar arrangement of collagen compared to control tissues which had diffuse collagen staining throughout the DE. In addition, indirect immunofluorescence (IIF) of skin substitute tissues showed the presence of elastin (red) in the DEs of cell-derived tissues, a major protein component of the human dermis that is responsible for skin resilience; elastin is not detected in control tissues. Nuclei in IIF samples were counterstained with Hoescht (blue). Bar = 100 µm.



5. Composition and structure of skin substitute tissues

Skin Substitute Properties	StrataGraft [®]	Cell-derived
Thickness (µm)	163 ± 12	191 ± 27
Dermal Cellularity (NHDF/cm ²)	2.2 ± 0.4 x10 ⁵	3.4 ± 0.5 x10 ⁵
Collagen Content (µg/cm ²)	437 ± 18	204 ± 61
Viability - MTT (O.D.)	0.90 ± 0.03	0.77 ± 0.10

Composition and structure of cell-derived skin substitutes were analyzed and compared to the properties of StrataGraft[®] skin tissues. **Tissue thickness** was averaged from readings at several points across the surface; n = 6 StrataGraft[®] tissues (2 batches); n = 10 Cell-derived tissues (3 batches). **Collagen density** was calculated using a colorimetric Sirius red assay (modified from [5]); n = 6 StrataGraft[®] tissues (2 batches); n = 11 Cell-derived tissues (3 batches). **Dermal cellularity** was calculated by removing and digesting the DE and performing direct cell counts; n = 6 StrataGraft[®] tissues (2 batches); n = 7 Cell-derived tissues (2 batches). **Skin substitute viability** was determined by MTT assay; n = 6 StrataGraft[®] tissues (2 batches); n = 11 Cell-derived tissues (3 batches). Significant **increases** or **decreases** (p<0.05) in the properties of cell-derived skin substitutes compared to StrataGraft[®] controls were determined by t-test.

6. Functional properties of skin substitute tissues

Skin Substitute Properties	StrataGraft [®]	Cell-derived	
Barrier Function	Initial DPM	95 ± 6	93 ± 2
	ΔDPM	164 ± 90	132 ± 55
Tensile Properties	Tensile Strength (kPa)	703 ± 120	831 ± 178
	Failure Strain (mm/mm)	1.66 ± 0.23	1.53 ± 0.16
VEGF secretion (pg/cm ² /24hr)	519 ± 37	630 ± 18	

Functional properties of cell-derived skin substitutes were analyzed and compared to the properties of StrataGraft[®] skin tissues. **Barrier function** was assessed as the average of four measurements taken across the surface; n = 6 StrataGraft[®] tissues (2 batches); n = 7 Cell-derived tissues (2 batches). **Uniaxial tensile properties** were measured on dogbone shaped tensile specimens (25 mm x 4 mm, LxW gage region) tested at a constant extension rate of 25mm/min with continuous hydration by PBS; n = 6 StrataGraft[®] tissues (2 batches); n = 11 Cell-derived tissues (3 batches). **Secretion of the pro-angiogenic factor VEGF** was determined by ELISA of conditioned medium; n = 3 StrataGraft[®] tissues (1 batch); n = 3 Cell-derived tissues (1 batch). Significant **increases** or **decreases** (p<0.05) in the properties of cell-derived skin substitutes compared to StrataGraft[®] controls were determined by t-test.

Discussion

Despite several significant differences in the dermal composition and structure, NHDF-derived DEs supported formation of mature skin substitutes containing fully-stratified epidermal layers with mechanical properties and barrier function comparable to tissues produced with gelled collagen DEs.

Elimination of exogenous collagen simplifies the manufacturing process and may facilitate the transition to a closed and automated production process, reducing treatment costs.

However, allogeneic cell sourcing is a significant challenge for tissue engineering applications, so it is critical that chosen cell seeding densities can be reasonably scaled to commercial production. With that in mind, this study employed significantly lower cell densities than have been previously reported [1,2].

Cell-derived skin substitutes in the current study required approximately twice the production time of StrataGraft[®] controls; further optimization will be necessary for commercial use.

Although DEs composed entirely of human cells and extracellular matrix may offer advantages *in vivo*, maintaining DE consistency will be critical. *De novo* tissue generation by NHDFs could introduce variability avoided by controlled collagen gel polymerization.

Conclusions

- Long-term culture of NHDFs can generate substantial dermal equivalent layers which support the production of fully cell-derived skin substitute tissues.
- Composition of NHDF-derived DEs more accurately reflects that of the normal human dermis, including matrix components, such as elastin, that are not observed in collagen gel DEs.
- Skin substitutes generated using NHDF-derived DEs demonstrated significant differences in tissue composition and structure compared to StrataGraft[®] controls, producing thicker tissues that contained more NHDFs, but significantly less collagen.
- Importantly, very few differences were seen in the functional tissue characteristics. All skin substitutes could be easily handled and meshed, provided similar mechanical properties and barrier function, and secreted wound healing factors, such as VEGF.
- Elimination of animal-derived collagen from tissue-engineered skin substitutes simplifies the manufacturing process, improves product safety, and may reduce immunological responses to the tissue.

References

1. Ahlfors et al. Biomaterials, 2007; 28(13): 2183-2191
2. Pouyani et al. In Vitro Cell Dev Biol Anim, 2009; 45(8): 430-441
3. Schurr et al., J of Trauma, 2009; 66(3): 866-874
4. Allen-Hoffmann et al., J of Inv Derm, 2000;114(3): 444-455
5. Tullberg-Reinert et al. Histochem Cell Biol, 1999; 112(4): 271-276

Acknowledgements

This work was supported in part by a small business grant from the NIH (NIA R44AG027625 to ARC).