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EXPERIMENTAL

Autologous Graft Thickness Affects Scar Contraction and Quality in a Porcine Excisional Wound Model

Rodney K. Chan, MD, FACS*†
Lloyd F. Rose, PhD*†
Jesse C. Wu, MS*†
David I. Tucker, DDS*†
Maren M. Chan, MD‡
Robert J. Christy, PhD*
Robert G. Hale, DDS*†
Kai P. Leung, PhD*†

Background: Texture, color, and durability are important characteristics to consider for skin replacement in conspicuous and/or mobile regions of the body such as the face, neck, and hands. Although autograft thickness is a known determinant of skin quality, few studies have correlated the subjective and objective characters of skin graft healing with their associated morphologic and cellular profiles. Defining these relationships may help guide development and evaluation of future skin replacement strategies.
Methods: Six-centimeter-diameter full-thickness wounds were created on the back of female Yorkshire pigs and covered by autografts of variable thicknesses. Skin quality was assessed on day 120 using an observer scar assessment score and objective determinations for scar contraction, erythema, pigmentation, and surface irregularities. Histological, histochemical, and immunohistochemical assessments were performed.
Results: Thick grafts demonstrated lower observer scar assessment score (better quality) and decreased erythema, pigmentation, and surface irregularities. Histologically, thin grafts resulted in scar-like collagen proliferation while thick grafts preserves the dermal architecture. Increased vascularity and prolonged and increased cellular infiltration were observed among thin grafts. In addition, thin grafts contained predominately dense collagen fibers, whereas thick grafts had loosely arranged collagen. α-Smooth muscle actin staining for myofibroblasts was observed earlier and persisted longer among thinner grafts.
Conclusions: Graft thickness is an important determinant of skin quality. High-quality skin replacements are associated with preserved collagen architecture, decreased neovascularization, and decreased inflammatory cellular infiltration. This model, using autologous skin as a metric of quality, may give a more informative analysis of emerging skin replacement strategies. *(Plast Reconstr Surg Glob Open 2015;3:e468; doi: 10.1097/GOX.0000000000000426; Published online 22 July 2015.)*

In burn patients, restoration of barrier function is paramount, as skin closure is a main determinant of survival.[1] Thus, in clinical care of deep partial-thickness and full-thickness burns, early excision is commonly followed by coverage with autologous partial-thickness skin grafts. One determinant of final skin quality is the thickness of autograft applied.[2] In functionally important areas of the face, neck, and hands, achievement of high-quality skin that permits joint movement, physiological functions, and cosme-

*From the *US Army Institute of Surgical Research, Fort Sam Houston, Tex.; †Dental and Trauma Research Detachment, US Army Institute of Surgical Research, Fort Sam Houston, Tex.; and ‡Department of Pathology and Area Laboratory Services, San Antonio Military Medical Center, Fort Sam Houston, Tex.*

Received for publication June 12, 2014; accepted May 28, 2015.

DOI: 10.1097/GOX.0000000000000426

sis is an important consideration and a thicker unmeshed graft is typically used.[3]

Although this practice results in better clinical outcomes, clinicians are hesitant to apply thick grafts because thicker skin requires a more ideal recipient wound bed for revascularization. Thicker grafts are also associated with higher donor-site morbidity and decrease the number of times a donor site can be reharvested. Applying thinner and more easily revascularized grafts increases the likelihood of incorporation but risks a less desirable outcome in terms of scar formation.

The long-term effect of low-quality skin replacement is function-limiting burn scar contracture. Surgical revision generally involves secondary scarred skin replacement using either autologous skin grafts or skin flaps. After initial injury and acute care, burn patients often must commit years to scar contracture revision, significantly delaying return to a normal functional lifestyle.

The ideal skin replacement therapy is one that, with the fewest possible operations, achieves as many functions of normal skin as possible. One of the gaps in achieving satisfactory results lies in our understanding of how graft thickness alters wound healing and resists the forces of contraction.

Therefore, a quantifiable and relevant preclinical model is important to evaluate autologous grafting techniques and to set standards for tissue-engineered skin substitutes. Using a porcine full-thickness wound model, we correlated the thickness of autograft skin with scar quality. Objective and subjective metrics of graft quality were further correlated with morphological, histological, and immunohistochemical measures. Our results present a relevant preclinical model for the assessment of the quality of skin replacement therapy and may guide development and evaluation of future skin substitutes.

MATERIALS AND METHODS

Animals

The use of 6-month-old crossbred female Yorkshire pigs (Midwest Research Swine, Gibbon, Minn.) was reviewed and approved by Institutional Animal Care and Use Committees at the United States Army Institute of Surgical Research (JBSA Fort Sam Houston, Tex.). All animals received care in strict compliance with the 2011 *Guide for the Care and Use of Laboratory Animals* by the National Research Council and were maintained in Association for Assessment and Accreditation of Laboratory Animal Care International–accredited facilities.

Disclosure: *The authors have no financial interest to declare in relation to the content of this article. This study is funded by the Department of Defense. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense. The Article Processing Charge was paid for by the US Army Medical Research and Material Command.*

Full-thickness Excision and Grafting

Five 6-cm wounds were marked (4 cm from each other and 2 cm from the spine) on each dorsal side of the animal with edges tattooed. To control for animal growth, 2 additional 6-cm unwounded circles were tattooed. Total wound area was less than 10% total body surface area. After full-thickness excision down to muscle fascia, wounds were grafted using autologous skin from a hind-limb donor site. Autografts were harvested using a Zimmer pneumatic dermatome (Zimmer Surgical, Dover, Ohio) and cut to fit defect. Eleven pigs were used in this study with autograft locations randomized among the following groups with n = 8 wounds: 6/1000th (150 μm), 12/1000th (300 μm), 20/1000th (500 μm), or 30/1000th (760 μm) of an inch grafts, and full-thickness skin graft. Full-thickness autograft skin was harvested from the original full-thickness wound creation and surgically defatted.

Observer Scar Assessment Scale

Using the observer portion of the POSAS (Patient and Observer Scar Assessment Scale), scars were examined by 4 unblinded observers. All observers were also involved in the surgery, with no additional personnel available to make blinded assessments. Vascularity (degree of redness), relief (surface irregularity), and mobility (graft adherence to underlying surface) were measured compared to normal skin and a score assigned for each parameter.[4] In short, selected variables were assigned a score from 1 to 10, with a score of 1 reflecting close to normal skin and 10 representing the worst possible score. The remaining scar categories were determined to be unreliable measures of scarring in our model or were already being assessed objectively.

Objective Graft Analyses

Graft dimensions were analyzed by ImageJ software (National Institute of Health, Bethesda, Md.) and calculated as a percentage of initial graft size corrected for animal growth. Direct measurement of graft elevation used a handheld laser image capture device (SilhouetteMobile, Aranz Medical, New Zealand) measuring wound area, volume, height,

and depth. Hemoglobin and melanin concentration were assessed with the Cortex Technology DSMII Colorimeter (CyberDerm, Broomall, Pa.) normalized to adjacent healthy skin.

Histology

On days 7, 14, 30, 60, 90, and 120 postgrafting, 12-mm punch biopsy samples of the graft were taken, fixed in 10% neutral-buffered formalin or Carnoy's solution (for mast cell staining), dehydrated, and embedded in paraffin. Five-micrometer-thick sections were deparaffinized and stained with hematoxylin-eosin, Verhoeff's Elastic Masson's Trichrome for collagen and elastin, picrosirius red for collagen, or Alcian blue for mast cells. All sections were photographed (Eclipse 55i and DS-Fi1, Nikon, Melville, N.Y.) under white light or plane-polarized light (picrosirius red stain). Histologic measurement of skin graft and scar thickness was performed on day 120. Mast cells were counted in 10 randomly selected high-power fields (×400) in the superficial dermis and results expressed as number of cells per field.

Immunohistochemistry

Tissues were cut into 5-μm-thick sections. After deparaffinization and hydration, antigen retrieval was performed and sections blocked with 10% normal goat serum for 1 hour at room temperature. Sections were then treated with primary antibodies, rabbit pAb α-smooth muscle actin (α-SMA) (Abcam, Cambridge, Mass.) and CD45 (Novus, Littleton, Colo.), overnight at 4°C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Primary antibody was detected with horseradish peroxidase–conjugated secondary antibody (goat anti-rabbit or goat anti-mouse) (Bio-Rad, Hercules, Calif.) followed by diaminobenzidine (DAB) development (Vector Lab, Burlingame, Calif.). Slides were observed under Nikon Eclipse 55i light microscope and photographs taken with a Nikon DS-Fi1 camera.

Statistical Analysis

Statistical differences for Observer Scar Assessment Scale (OSAS) scoring, colorimetry, and graft contraction were determined by two-way analysis of variance with Tukey-Kramer adjustment, or nonparametric comparison for all pairs using Steel-Dwass method when appropriate, using JMP statistics software (SAS, Cary, N.C.). Results are presented as mean ± standard error of the mean with $P < 0.05$ considered significant.

This study has been conducted in compliance with the Animal Welfare Act, the Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals.

	6/1000th A	12/1000th B	20/1000th C	30/1000th D	FTSG E	P-Value
% Initial Graft Size	52.49% ± 3.75%	65.93% ± 5.52%	86.54% ± 4.84%	86.33% ± 2.54%	99.87% ± 7.42%	AE: <0.0001 BE: 0.0005 AD: 0.0018 AC: 0.0011
Graft Elevation	1.04mm ± 0.26mm	0.44mm ± 0.27mm	0.23mm ± 0.46mm	-0.64mm ± 0.45mm	0.38mm ± 0.47mm	AD: 0.0374
Erythema	11.45 ± 0.54	8.46 ± 1.26	6.58 ± 1.16	4.34 ± 0.63	6.70 ± 1.34	AC: 0.0325 AD: 0.0011 AE: 0.0315
Melanin	39.88 ± 1.57	37.43 ± 1.19	32.70 ± 1.24	32.62 ± 0.83	33.20 ± 0.87	AC: 0.0012 AD: 0.0184 AE: 0.0021 BC: 0.0432
POSAS Total	22.36 ± 1.86	21.96 ± 1.71	17.65 ± 2.58	14.81 ± 2.22	14.56 ± 1.44	
• POSAS Vascularity	6.82 ± 0.47	5.62 ± 0.63	4.93 ± 0.62	3.46 ± 0.36	4.19 ± 0.68	AD: 0.0022 AE: 0.0308
• POSAS Thickness	3.15 ± 0.45	3.13 ± 0.24	1.94 ± 0.74	1.75 ± 0.57	3.08 ± 0.45	
• POSAS Relief	5.80 ± 0.72	5.71 ± 0.70	4.60 ± 0.93	4.68 ± 0.76	2.53 ± 0.31	AE: 0.0460 BE: 0.0456
• POSAS Mobility	6.59 ± 0.55	6.38 ± 0.56	5.47 ± 0.55	4.92 ± 0.89	4.75 ± 0.63	

Fig. 1. Summary of graft contraction, graft elevation, erythema, melanin, and Patient and Observer Scar Assessment Scale (POSAS) measurements of various thickness skin grafts on day 120 postgrafting. The 2-letter combinations under "*P*-value" refer to comparisons between the designated columns. Thus, "AE: <0.0001" indicates that the calculated *P* value between contraction of a 6/1000th (150 μm) graft (column A) and a full-thickness skin graft (FTSG; column E) is 0.0010.

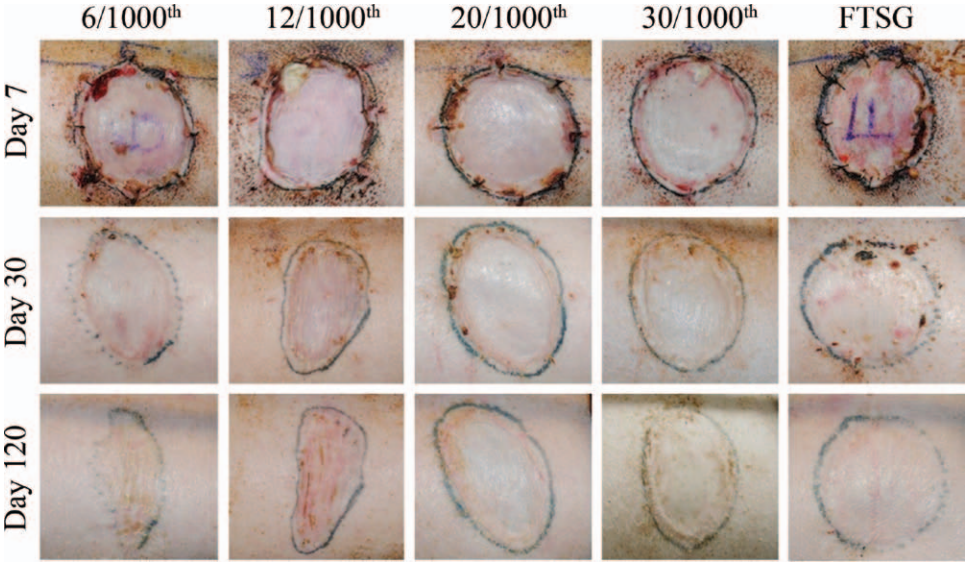

Fig. 2. Graft size of various thickness skin grafts monitored over 120 days. Thinner grafts have contracted significantly over time while thicker grafts have closely preserved their original size. FTSG indicates full-thickness skin graft.

RESULTS

Graft Thickness Correlates with Subjective Measures of Skin Quality

Subjective assessments of graft healing were performed on day 120 postgrafting for vascularity, thickness, relief, and mobility (OSAS; Fig. 1). Thinner grafts (6 or 12/1000th of an inch, or 150 or 300 μm, respectively) are well vascularized with a significantly redder appearance and more surface irregularities than thicker grafts (20 or 30/1000th of an inch, or 500 or 760 μm, respectively, and FTSG). Although there seems to be no difference between the groups in terms of thickness, thin grafts tend to have higher mobility scores, indicating that the skin graft appears more adherent and immobile relative to the underlying surface. Thin grafts also have significantly higher hemoglobin concentration (higher vascularity) based on the erythema measurement of redness, consistent with subjective measurements of graft vascularity, and have significantly higher melanin concentration (increased pigmentation). Taken together, thicker grafts have lower total OSAS scores compared with thinner grafts.

Graft Thickness Correlates with Objective Measures of Skin Quality

Objective assessments of contraction, graft elevation, and colorimetry measurements were taken over

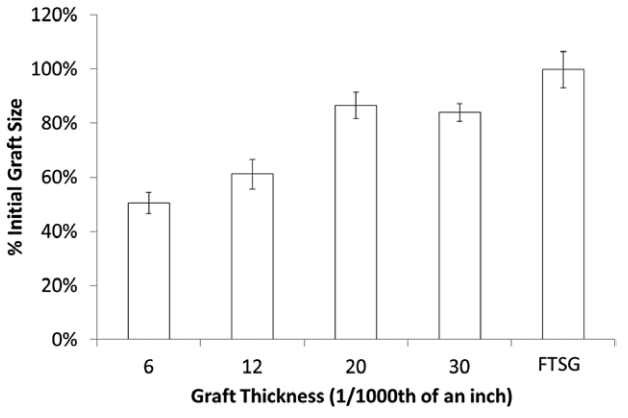

Fig. 3. Size of various thickness skin grafts on day 120 postgrafting. Thin grafts have contracted significantly as measured by the percentage of their original size compared with thicker grafts. Statistical analysis is shown in Figure 1. FTSG indicates full-thickness skin graft.

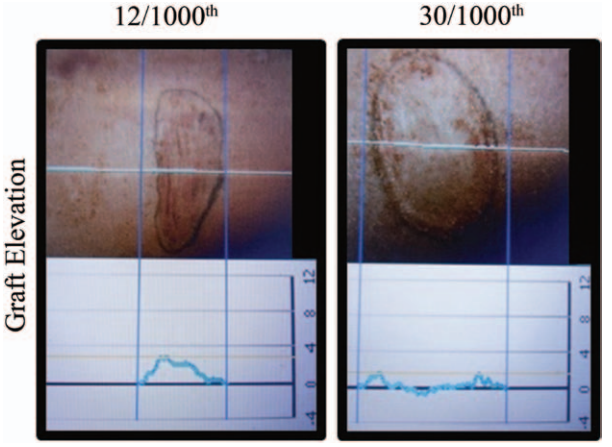

Fig. 4. A graphical representation of graft elevation measurements by SilhouetteMobile. The thin graft shows more contraction (top picture) and higher elevation (bottom graph) compared with the thick graft.

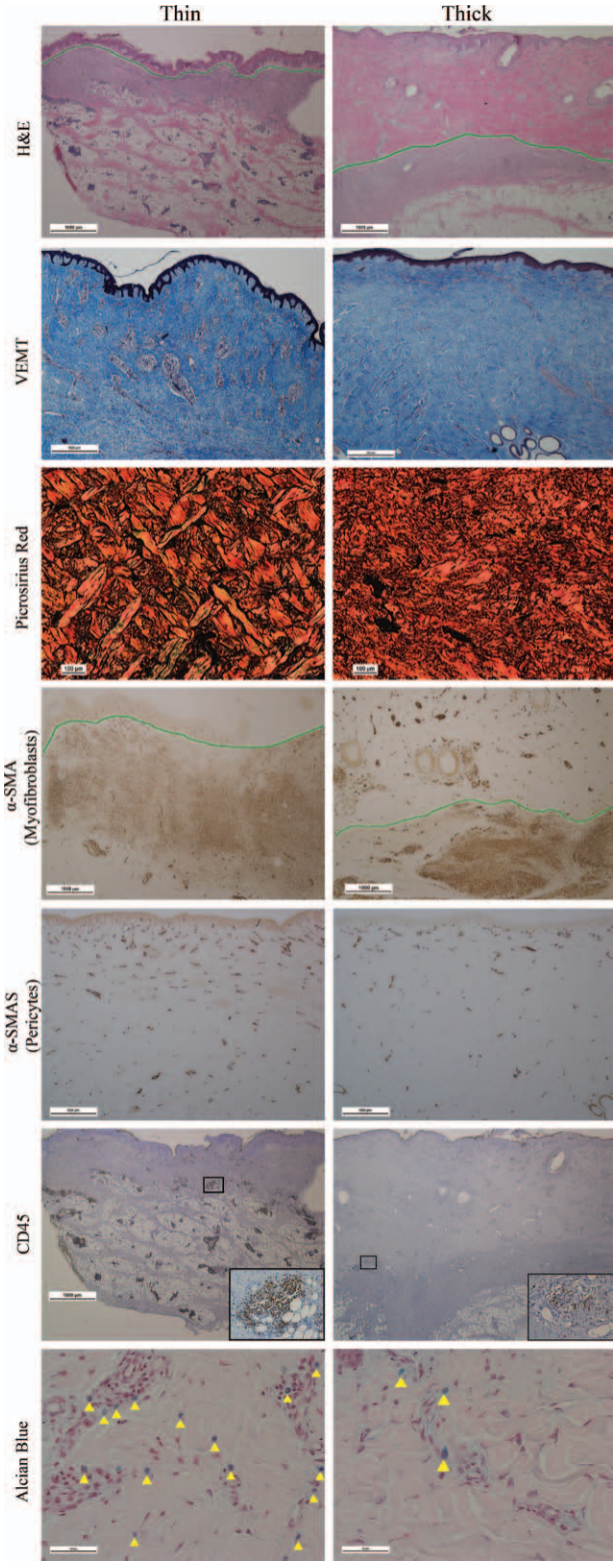

Fig. 5. Histological comparison between thin (A) (6/1000th of an inch, 150 μm) and thick (B) (full-thickness skin graft) grafts. H&E: Hematoxylin and eosin stain on day 7 postgrafting. The green line depicts the junction between graft and host tissue (×20). VEMT: Verhoeff's Elastic Masson's Trichrome stain for collagen on day 120 (×40). Picrosirius Red: Collagen

120 days. Autograft thickness significantly affects wound contraction (Fig. 2). Analysis of the resulting graft size is presented as the % initial graft size (Figs. 1, 3), with thicker grafts contracted significantly less (higher % initial graft size) than thinner grafts. Thicker grafts were also less elevated (Fig. 4), indicating less surface deformation from contraction.

Thicker Grafts Have Distinct Histological Characteristics

Hematoxylin-eosin staining at day 7 postgrafting showed marked infiltration of inflammatory cells at the graft-host junction (Fig. 5). Qualitatively, this inflammatory infiltration was greater in magnitude and persisted for a longer period of time in thin grafts (data not shown). Furthermore, this granulating layer was replaced with disorganized collagen in thin grafts while thick grafts preserved a native collagen architecture. Collagen staining in thin grafts on day 120 demonstrated persistent inflammation marked by mixed inflammatory infiltrates (lymphocytes, plasmacytes, neutrophils) and thin, dense, immature collagen laden with many fibrocytes. In thick grafts, the collagen was close to normal, consisting of large, loosely packed, evenly spaced bundles. Preservation of skin appendages, absence of inflammation, and normal amounts of fibrocytes were also observed. It is well known that as fiber density increases, picrosirius color changes; and as wound healing progresses, collagen fiber density increases.[5] Picrosirius red staining of thin grafts on day 120 showed predominately densely packed thin collagen fibers (red/orange), consistent with scar collagen maturation. By contrast, thick grafts contained loosely spaced, thick bundles of interwoven collagen (orange/yellow) resembling normal skin. Grafts also demonstrated a distinct CD45 immunohistochemical profile on day 7. Throughout thin grafts, leukocyte infiltration was dense and pervasive, whereas in the thick grafts, inflammatory infiltrate was minimal, with few CD45+ cells. α-SMA staining on day 14 in thin grafts showed a thicker layer of myofibroblasts

Fig. 5. (*Continued*). stain on day 120 (×100). Thick graft consists of large, interwoven, and loosely spaced collagen bundles, whereas thin graft contains densely packed thin collagen fibers. CD45: On day 7, leukocyte infiltration is dense (×400) and pervasive (×20) in the thin graft compared with the thick graft. α-SMA (myofibroblasts): Myofibroblast infiltration in host tissue on day 14 postgrafting (×20). The brown myofibroblast layer is present below the graft-host junction (green line). α-SMA (pericytes): Vascular network is noted primarily at the superficial dermis (day 120, ×40). Alcian Blue: Mast cells stain blue (yellow arrowheads) and are located primarily at the superficial dermis (day 120, ×400).

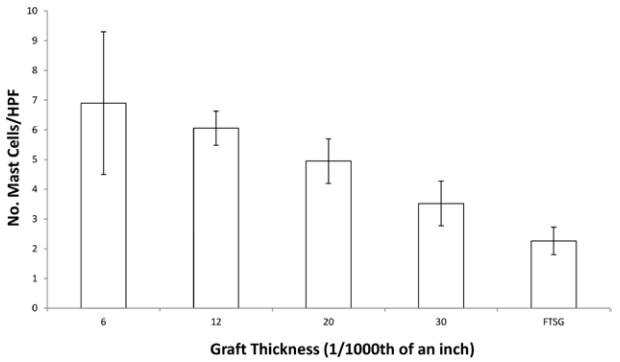

Fig. 6. The number of mast cells was counted for each group on day 120. The thicker graft, especially the full-thickness skin graft (FTSG), has fewer mast cells compared with the thinner graft.

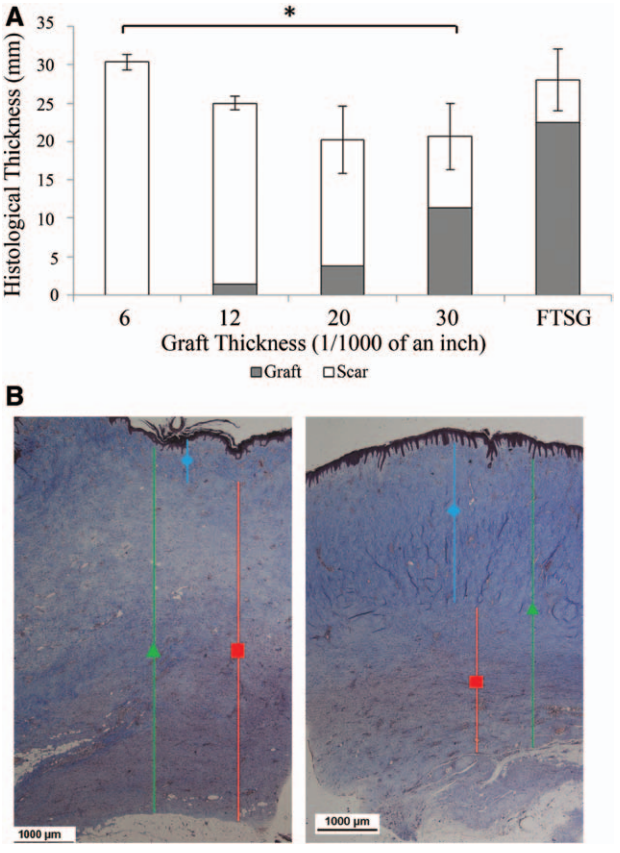

Fig. 7. Histological comparison of graft, scar, and total thickness between each autograft group on day 120. A, The graph shows an inverse relationship between graft thickness and scar thickness. B, Histological comparison between a thin (12/1000th of an inch, 300 µm) and a thick (30/1000th of an inch, 760 µm) graft. The blue line marks the original autograft, red line marks the scar, and the green dashed line marks the total dermal thickness. Thinner grafts have a thicker scar compared with thicker grafts. FTSG indicates full-thickness skin graft.

compared with thick grafts. Thin grafts also appeared to be hypervascular compared with thick grafts, as shown by dense pericyte staining around blood vessels on day 120. Mast cell staining revealed that thin grafts also have more mast cells (yellow arrowheads, Figs. 5, 6) than thick grafts on day 120. Objective measurement of graft and scar thickness was performed histologically (Fig. 7), revealing a direct correlation with resulting skin quality and an inverse relationship with scar thickness.

DISCUSSION

Autologous partial-thickness skin is the standard in skin replacement therapy. Although it has many advantages, we are far from an ideal therapy. When used to resurface an area where mobility or aesthetics is important, coverage with a thin partial-thickness autograft often results in a thick, nonpliable scar. In the quest for better skin replacement, it is important that a quantifiable and relevant preclinical model exists with which to evaluate and guide development of skin substitutes and that the model accurately reflects treatment and healing responses of human wounds. In this study, we describe a porcine model of full-thickness wounds, where a gradation of skin qualities was created experimentally using variable thickness autografts. Multiple methods to quantify skin quality were correlated with morphological, histochemical, and immunohistochemical characteristics.

The significance of this study lies in the characterization of a novel preclinical model that mirrors problematic clinical scenarios: durable coverage of full-thickness skin defects using skin grafts or skin substitutes. This model may be used to determine the value of emerging skin substitutes compared with the current standard of care (Fig. 8). For example, a hypothetical experimental skin substitute covered with a 12/1000th inch split thickness skin graft, yielding a 20% contraction rate, may be compared with the standard-of-care skin graft (40% contraction rate; Fig. 3), allowing an assessment of the experimental skin substitute as "overperforming" (green).

Equally important, this study establishes correlations between subjective and objective measures of skin quality, using OSAS to subjectively assess the quality of skin. The total OSAS demonstrated differences among the various graft thicknesses. Because the pigs were essentially white, we omitted pigmentation scoring. Although OSAS is cumbersome and requires education and calibration of observers, it facilitates comparison with published reports. However, an equivalent objective scoring system

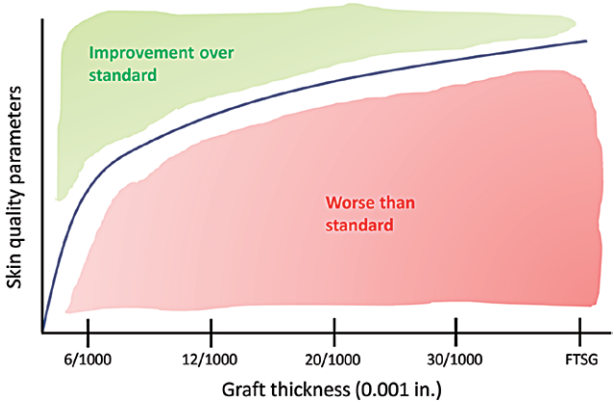

Fig. 8. The merit of current and emerging treatments or treatment strategies can be compared with the standards established by this model. The *y* axis can be any of the measures we have used for determining skin quality (ie, contraction and erythema). For example, if the standard contraction for a 12/1000th inch (300 μm) graft is 40%, then a skin substitute that yields 20% contraction would be an overperformer compared with the standard (green). If the same product has 50% contraction, then the product is an underperformer (red). FTSG indicates full-thickness skin graft.

is preferred. Indeed, this model permits objective validation of our subjective assessments. Although critics may argue that quality cannot be quantified, erythema, pigmentation, thickness, relief, and mobility are all quantifiable traits and taken together may represent a better objective scar scale for future preclinical and clinical studies.

- Using colorimetry,[6,7] we have demonstrated significant differences in erythema between thin and thick grafts that correlate with OSAS subjective assessments of vascularity. This is further confirmed by increased vessel density in thin grafts, which is demonstrated by increased pericyte staining. Although blood flow is clearly important for skin graft survival, hypervascularity is associated with lower quality skin and worse scar.[8–10]
- Objective measurements of pigmentation also demonstrated significantly more melanin in thinner grafts. Indeed, upregulation of melanocytes is a natural response to injury.[11] Therefore, it is not surprising that thin grafts, with less reconstitution of the dermis and worse scarring, have more melanin.
- Subjective assessment of scar thickness did not correlate well with graft thickness. Staining with Verhoeff's Elastic Masson's Trichrome allowed us to distinguish between skin graft height, scar height, and total skin height (Fig. 7). Graft thickness is inversely proportional to the amount of scar formed. The prolific collagen deposition among thin grafts is consistent with our clinical observation that application of a thin skin graft results in low-quality skin that is thick and stiff. Enhanced collagen deposition corresponds with increased inflammatory cellular infiltration, supporting our speculation that thinner grafts induce rampant inflammation that may directly or indirectly contribute to a robust fibrotic response. The amount of fibrosis observed may simply be a host response that takes place at the junction of skin graft and host tissue; however, thicker grafts have a lower inflammatory infiltrate and the scar formed under the graft is minimal. We did not attempt to measure skin or scar thickness noninvasively, but for future studies, ultrasound may be a useful modality.[12–14]
- Relief, the degree of surface irregularity relative to surrounding skin measured on day 120, was more pronounced in thinner grafts. A significant difference was observed between 6 and 30/1000th (150 μm and 760 μm) groups but not between other groups. This may result from scarring at the graft edge affecting average graft height or from lack of statistical power. Currently, subjective assessment is more correlative with quality than objective measurements.
- Objective assessment of stiffness/suppleness using a durometer correlates well with clinical assessment of pliability[7] although we found the readouts to be variable. Using OSAS we found thin grafts to be more stiff and immobile, in agreement with previous reports that because humans lack a panniculus carnosus, grafting directly onto fascia results in reduced movement of the graft over underlying tissues.[15,16] We have transitioned to using a cutometer to more accurately and objectively measure mobility but do not have enough data to draw conclusions.
- Contraction is a particularly meaningful measurement for assessment of skin graft and skin substitute quality, as the preservation of adjacent anatomy is so important for face and hands. Indeed, this was a highly significant variable when comparing the various groups. Though not as easily measured in a human clinical trial where permanent tattooing of the wound edges would be less acceptable, a tracing method that relies on adjacent immobile points may be used.[17]

Few reports have correlated histological and immunohistological characteristics of final skin quality with corresponding subjective and objective characteristics. We found that collagen fiber characteristics and the level of α-SMA expression correlate with graft thickness and skin quality. Others have noted its correlation with scar thickness and contraction.[18]

Collagen undergoes rapid maturation in fibrosis: fiber birefringence intensity increases, and polarization color changes as fiber density increases.[5,19] Our results show increased collagen density and a lack of collagen fiber organization in thin grafts, consistent with scar collagen maturation. By comparison, collagen in thick grafts is more organized and less dense, resembling normal skin, suggesting that thin grafts develop a thick disorganized scar by excessive synthesis of collagen, decreased matrix degradation, or both.

We noted increased numbers of mast cells in thin grafts, correlating with the worst scar. Mast cells stimulate type I collagen synthesis in fibroblasts in vitro,[20] and excessive accumulation and deposition of type I collagen is associated with dermal fibrosis.[21–24] Hypertrophic scars in both humans and Duroc pigs have been associated with high numbers of mast cells,[25] whereas fewer mast cells are observed in fetal or oral mucosal wounds compared with scar-forming adult dermal wounds.[26–28]

Inflammatory activation after severe burn injury is well documented, and burn is an independent predictor of scarring.[29] Several factors increase theisk of skin graft contraction, including extensive body surface involvement, thin grafts, and open wound beds,[30] all of which contribute to inflammatory activation. Indeed, we have shown that thin skin grafts, which result in greater contraction, elicit a strong cellular inflammatory infiltration at the graft-host junction. Infiltration of inflammatory cells in the early wound has been strongly linked to fibrogenesis and hypertrophic scarring.[31] Delayed eschar excision and delayed wound coverage, both seen among large surface area burns and especially among combat casualties, result in increased inflammation and graft contraction.[32] The use of negative pressure wound dressings in burns[33] can further elicit inflammation through a foreign body response.[34]

CONCLUSIONS

Using skin grafts of various thicknesses, we have established a model that may serve to evaluate products and methods of skin substitution. We have found objective measurements of erythema, pigmentation, and contraction to be highly quantitative and reliable, although subjective measurements are still necessary at this time for relief and mobility. Persistence and magnitude of inflammatory cellular infiltrate, levels of α-SMA expression, presence of mast cells, and the quality of collagen correlate with skin quality. These findings could facilitate the development of skin substitutes to achieve higher quality skin and wound closure while minimizing donor-site morbidity.

Rodney K. Chan, MD
US Army Institute of Surgical Research
3698 Chambers Pass, Ste B
JBSA Fort Sam Houston, TX 78234
E-mail: rodney.k.chan.ctr@mail.mil

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