Characterization of a Cryopreserved Split-Thickness Human Skin Allograft—TheraSkin

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ABSTRACT
OBJECTIVE: The purpose of this study was to examine the characteristics of a cryopreserved split-thickness skin allograft produced from donated human skin and compare it with fresh, unprocessed human split-thickness skin.

BACKGROUND: Cutaneous wound healing is a complex and organized process, where the body re-establishes the integrity of the injured tissue. However, chronic wounds, such as diabetic or venous stasis ulcers, are difficult to manage and often require advanced biologics to facilitate healing. An ideal wound care product is able to directly influence wound healing by introducing biocompatible extracellular matrices, growth factors, and viable cells to the wound bed.

MATERIALS AND METHODS: TheraSkin (processed by LifeNet Health, Virginia Beach, Virginia, and distributed by Soluble Systems, Newport News, Virginia) is a minimally manipulated, cryopreserved split-thickness human skin allograft, which contains natural extracellular matrices, native growth factors, and viable cells. The authors characterized TheraSkin in terms of the collagen and growth factor composition using ELISA, percentage of apoptotic cells using TUNEL analysis, and cellular viability using alamarBlue assay (Thermo Fisher Scientific, Waltham, Massachusetts), and compared these characteristics with fresh, unprocessed human split-thickness skin.

RESULTS: It was found that the amount of the type I and type III collagen, as well as the ratio of type I to type III collagen in TheraSkin, is equivalent to fresh unprocessed human split-thickness skin. Similar quantities of vascular endothelial growth factor, insulinlike growth factor 1, fibroblast growth factor 2, and transforming growth factor β1 were detected in TheraSkin and fresh human skin. The average percent of apoptotic cells was 34.3% and 3.1% for TheraSkin and fresh skin.

CONCLUSIONS: Cellular viability was demonstrated in both TheraSkin and fresh skin.

KEYWORDS: human skin allograft, living skin equivalent, venous leg ulcer, diabetic foot ulcer

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INTRODUCTION
More than $25 billion is spent annually on the treatment of chronic wounds, and the burden is growing rapidly because of increasing healthcare costs, an aging population, and a sharp increase in the incidence of diabetes and obesity. Nonhealing wounds become chronic for a variety of reasons. The classic example of the diabetic foot ulcer requires the orchestration of numerous native and environmental factors to heal; however, 3 main factors work against successful wound healing. High levels of bacteria and the presence of necrotic tissue within the wound bed perpetually stimulate macrophages, keeping the wounds in a state of constant inflammation rather than repair. In addition, mechanical forces associated with walking traumatize the delicate neodermis as it forms, further delaying wounds from healing. A multitude of factors are involved in the wound healing cascade, many of which occur at the molecular level. Growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) produced by macrophages help to attract fibroblasts and keratinocytes to the wound site. Fibroblasts form and secrete extracellular matrix (ECM) components, such as hyaluronan and collagen, which serve as a scaffold to support the migration of cells across the wound surface. The ECM acts as a storage site for growth factors that can trigger angiogenesis, mitogenesis, and chemotaxis to further perpetuate the healing response. When the vascular supply is sufficient, granulation tissue develops.
The wound bed is also challenged by environmental factors, such as biofilm, a dense polysaccharide layer that protects and allows bacteria to flourish in the wound bed. Hydration is critical for healing wounds, and desiccation and/or maceration results in further deterioration of the wound bed, making it more difficult for epithelialization to occur.

Ultimately, chronic wounds require a support network of exogenous and endogenous factors to achieve closure. Bacterial loads can be controlled with antibiotics and debridement. When necessary, vascular supply can be improved with surgical techniques, and mechanical forces can be controlled with casting, special shoes, or prolonged non-weight bearing. However, once conditions have been optimized, it becomes a matter of coordination of the molecular factors to achieve wound closure. Raw materials, such as collagen and growth factors, become essential to effective wound closure, and this is frequently the stage where closure fails.

An interesting observation made by Sheehan et al revealed that wounds that do not show rapid progression during the first 4 weeks of treatment will rarely achieve full closure by week 12. Conversely, wounds that show at least 50% closure during the first 4 weeks will achieve closure by week 12 approximately 58% of the time. The idea of triggering rapid progression of healing during the first 4 weeks of treatment has led to the adoption of advanced biologics earlier in the treatment process. Advanced biologic materials can include an ECM that provides growth factors, collagen, and living cells. All 3 of these elements are critical in the treatment strategy of chronic wounds; these elements are also the focus of the current study.

For wound treatments, the split-thickness skin autograft is the criterion standard. Skin is harvested from a section of the patient (donor site) and transferred to the wound site (recipient site). This approach provides patients with their own living cells, collagen, and growth factors, but can introduce substantial morbidity, risk of infection, and pain at the donor site. In many cases, there is the problem of dissimilar skin between the donor and recipient sites. Also, each split-thickness skin graft requires a trip to the operating room, something that is usually highly impractical and very expensive to do on a repetitive basis, particularly in the operating room, something that is usually highly impractical and very expensive to do on a repetitive basis, particularly in the patient population where chronic wounds typically occur.

In response to the need to provide ECM, growth factors, and living cells to the wound while avoiding the cost and complications of patient surgeries, several treatment options have emerged. Biologically active skin substitutes, such as Dermagraft (Organogenesis, Canton, Massachusetts) and Apligraf (Organogenesis), contain collagen and living cells but differ substantially from skin. This has led clinicians to seek more complete treatment options.

TheraSkin is a cryopreserved split-thickness skin allograft (CSSA) produced from donated human skin (processed by LifeNet Health, Virginia Beach, Virginia, and distributed by Soluble Systems, Newport News, Virginia). TheraSkin was designed to deliver these essential biologically active compounds to the wound bed in a convenient office setting. The skin is procured from authorized tissue donors who meet the strict safety criteria inclusive of microbiologic and virology panels mandated by the FDA, the American Association of Tissue Banks, and the manufacturer. Following a thorough disinfection process, the skin graft is cryopreserved for storage and distribution. It meets the criteria required by the FDA to be regulated as a Human Cell and Tissue Product (361 HCT/P) and is not classified as a medical device or biologic because it is minimally manipulated.

The objective of the current study was to characterize the collagen and growth factor composition, percentage of apoptotic cells, and cellular viability in CSSA that has gone through routine procurement, processing, disinfection, cryopreservation, and thawing in preparation for application at the wound site relative to fresh human skin controls.

**MATERIALS AND METHODS**

**Tissue**

All split-thickness skin was recovered from deceased donors with research authorization. Unprocessed fresh skin was used as the positive control. Cryopreserved split-thickness skin allograft was processed, disinfected, cryopreserved, and stored per standard procedures. The sample pieces taken were randomly allocated to be analyzed as either fresh or frozen. Not all samples were matched pairs, as there was not always the same number of specimens available from each donor. Fresh allografts were analyzed immediately, and CSSAs were analyzed following the standard processing, including disinfection, cleansing cryopreservation, and rewarming to room temperature. Harvested samples were assessed using 3 techniques: TdT-dUTP nick-end labeling (TUNEL), alamarBlue cell viability assay, and enzyme-linked immunosorbent assay (ELISA) (Thermo Fisher Scientific, Waltham, Massachusetts). The 3 techniques gave different types of information so that the fresh and prepared samples could be compared.

The TUNEL technique determines the percentage of apoptotic cells present. Hematoxylin-eosin (H&E) staining and DermaTACS In Situ Apoptosis Detection Kit (Trevigen, Inc, Gaithersburg, Maryland) for TUNEL staining were used to measure the percentage of apoptotic cells. Apoptosis refers to cells that are undergoing enzymatic degradation and are reaching the end of their lifespan. These cells are useful in that they provide proteins to the wound bed but are not actively replicative or producing new collagen and growth factors.

alamarBlue is used to assess cellular viability and proliferation of cells. It is based on mitochondrial activity. Viable, proliferative cells play a critical role in generation of growth factors and
Collagen production. Cellular viability was assessed with an alamarBlue cell viability assay.

An ELISA was performed to quantify human types I, III, and IV collagens, as well as growth factors found in each sample. A summary of the tests performed, donors used, and period of time for cryopreservation of the CSSA prior to testing can be found in Table 1.

**Collagen Quantification**

Both CSSA and fresh skin samples, each gathered from 2 different donors, were cut into small pieces and extracted in 0.5 M acetic acid with 1 mg/mL pepsin at a concentration of 50 mg tissue/mL on a shaker for 24 ± 2 hours at room temperature. Digested samples were centrifuged at 2000 relative centrifugal force (RCF) for 3 minutes, followed by 10,000 RCF for 5 minutes. The supernatants were recovered and stored at -80°C for analysis by ELISA. The amounts of human types I and IV collagen were quantified using ELISA kits from Cosmo Bio Incorporation (Carlsbad, California) and Echelon Biosciences Incorporation (Salt Lake City, Utah), respectively. Type III collagen was quantified by indirect ELISA using a mouse anti–type III collagen monoclonal capture antibody (clone IE7-D7; EMD Millipore Incorporation, Billerica, Massachusetts). All collagen quantities were normalized to tissue wet weight. The average quantity of each type collagen was calculated. In addition, the ratios of types I and III collagen in both CSSA and fresh skin were calculated.

Both CSSA and fresh skin samples, each from 2 different donors, were cut into small pieces and extracted in T-PER tissue protein extraction reagent (Thermo Scientific, Waltham, Massachusetts) at a concentration of 50 mg tissue/mL. The samples were extracted in a water bath sonicator for 15 minutes at room temperature, followed by gentle shaking for 22 hours at 4°C. Digested samples were centrifuged at 2000 RCF for 3 minutes, followed by 10,000 RCF for 5 minutes. The supernatants were recovered and stored at -80°C. The samples were assayed for insulin-like growth factor 1 (IGF-1), fibroblast growth factor 2 (FGF-2), TGF-β1, PDGF-AA, PDGF-BB, and PDGF-AB at the μQUANT Core Facility, Institute of Human Virology (Baltimore, Maryland) using Quantikine ELISA kits (R&D Systems, Inc, Minneapolis, Minnesota). In the case of vascular endothelial growth factor (VEGF), an ELISA kit from Ray Biotech (Norcross, Georgia) was utilized. All growth factor quantities were normalized to tissue wet weight. The average quantity of each growth factor in tissue from 2 donors was calculated.

**Tissue Preparation for Histology**

Three random samples of CSSA, each from 3 different donors with research authorization, were prepared for subsequent H&E and TUNEL analysis. The product storage time at the time of testing ranged from 2 to 13 months. Each graft was thawed and prepared according to the manufacturer’s instructions for clinical application. Three samples (1 × 0.5 cm each) were obtained from diverse areas of each graft, fixed in formalin, and embedded in paraffin for apoptotic cell analysis. The positive control fresh skin was obtained during routine skin recovery and immediately placed into 10% neutral buffered formalin for apoptotic cell analysis. All tissues were embedded on the long axis to facilitate preparation of slides containing cross sections of all anatomical layers of the skin. Five-micron-thick sections were prepared for H&E and TUNEL staining.

**TUNEL Staining**

The TUNEL staining was performed using the CardioTACS TUNEL kits ( Trevigen, Inc) with modifications based on Trevigen’s DermaTACS Procedure for skin tissue, to improve reagent permeation into the tissue. A positive TUNEL assay control was induced using the kit nuclease, and a negative control was produced by omitting the terminal deoxynucleotidyl transferase enzyme from the reaction mix. Two sections per slide were subjected to TUNEL staining.

**Quantitative Analysis for Apoptotic Cells**

For each stained slide (TUNEL and H&E), the best quality tissue section was selected and imaged using National Institutes of Health (NIH) Image Pro. For each tissue sample, the H&E images were used to calculate the total number of cells (cell density), and the TUNEL images were used to calculate the number of apoptotic cells utilizing a computerized thresholding method (NIH Scion Image for Windows software, version Beta 4.0.2.7). The volume of the photographed area was calculated by calibrating the viewed area against a micrometer and multiplying by the thickness of the tissue section (5 μm). An average cell

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; TUNEL, TdT dUTP nick-end labeling.
density and number of apoptotic cells were estimated per volume, both within (n = 3) and among the donors. For each sample, the percentage of apoptotic cells was calculated as follows: percentage of apoptotic cells = [(total number of cells by H&E – total number of TUNEL negative cells) / total number of cells by H&E] × 100. The average cell density and number of apoptotic cells among CSSA donors were compared with those in the fresh, unprocessed skin control.

Cellular Viability Assessment

alamarBlue cell viability reagent, which functions as an indicator of mitochondria metabolic activity, was used to quantitatively measure the viability and proliferation of cells. Cryopreserved split-thickness skin allograft samples from research authorized donors (n = 5) and fresh skin samples from different research authorized donors (n = 5) were used for viability assessment. All CSSA and fresh skin samples were obtained from donors with warm ischemic times (WITs) of less than 24 hours. Cryopreserved split-thickness skin allograft samples were thawed according to the manufacturer’s instructions. Fresh skin samples were stored in RPMI media at 4°C for no more than 3 days after recovery and tested. For both CSSA and fresh skin samples, 6 biopsy punches 12 mm in diameter were obtained from the skin of each donor. The thickness of the tissue was measured and averaged to calculate the volume of each biopsy punch. Punched skin samples were immersed and incubated in keratinocyte growth media (ATCC, Manassas, Virginia) containing 10% alamarBlue cell viability reagent for 20 hours at 37°C in an incubator with 5% CO2. After the incubation, the incubated alamarBlue media was transferred to a 96-well plate, and the fluorescence was measured using a microplate reader (Fluoroskan Ascent; Thermo Scientific) using an excitation wavelength of 544 nm and an emission wavelength of 592 nm. Relative fluorescence units (RFUs) of the reagent blank were subtracted from the sample RFUs to calculate a sample-specific RFU. The final RFUs were normalized to the volume of alamarBlue media added to each biopsy punch and the volume of the biopsy punch.

RESULTS

Collagen Quantification

The average quantity of human type I collagen in CSSA and fresh skin was 56.6 and 43.9 mg/g of tissue, respectively (Table 2). The average amount of human type III collagen in CSSA and fresh skin was 5.8 and 4.1 mg/g of tissue. The average ratio of type I to type III collagen for CSSA was 9.9, which was comparable to the ratio for fresh human skin (10.9). The average quantity of human type IV collagen in CSSA and fresh skin was 0.03 and 0.02 mg/g of tissue, respectively. Therefore, compared with fresh skin, CSSA maintains similar quantities of collagens and a similar ratio of types I and III collagen.

Growth Factor Quantification

Detectable quantities of VEGF, IGF-1, FGF-2, and TGF-β1 from CSSA and fresh skin were measured and calculated as picogram per gram of tissue (Table 3). High quantities of IGF-1, FGF-2, and TGF-β1 were present in both CSSA and fresh skin. The amount of VEGF present in both CSSA and fresh skin was...
low. All ELISA assays showed PDGF-AA, PDGF-BB, and PDGF-AB were below detectable quantities. In general, similar detectable quantities of growth factors were present in both CSSA and fresh skin.

### Apoptotic Cell Assessment

The calculated average total cell density and apoptotic cell density for tissue from each CSSA donor and fresh donor are summarized in Table 4. In general, more apoptotic cells were found in CSSA (Figure 1) compared with fresh skin (Figure 2). The average number of apoptotic cells in tissue from the 3 CSSA donors was 13,479 ± 14,015 per mm³, whereas the number of apoptotic cells in the fresh skin tissue was 1238 ± 643 per mm³. The percentage of apoptotic cells was approximately 34% and 3% for CSSA and fresh skin, respectively. The longer WIT, which is the time from asystole until the skin was placed into cold transport solution, may lead to more apoptotic cells over time.

### Cellular Viability Assessment

Cryopreserved split-thickness skin allograft and fresh skin samples incubated for 20 hours in media containing 10% alamarBlue reagent demonstrated that the normalized RFUs for CSSA from 5 donors varied between 8.14 and 21.62, and the average was 15.13 ± 2.85 (mean ± SEM) (Figure 3). The normalized RFUs measured for the fresh skin from 5 donors varied between 27.81 and 53.04, and the average was 37.80 ± 4.81 (mean ± SEM). The average viability of CSSA is approximately 40% that of the fresh skin based on normalized RFUs.

### DISCUSSION

In this study, it was demonstrated that TheraSkin, a minimally manipulated cryopreserved human skin allograft, maintains the natural extracellular matrices, native growth factors, and viable cells. Cryopreserved split-thickness skin allograft is unique among the advanced cellular and tissue-based products in that it contains both the epidermal and dermal layers that are naturally present in human skin.

In chronic wounds, abnormalities or deficiencies in ECMs, suboptimal or disrupted ECM and growth factor interactions, increased levels of inflammatory cells, and high levels of matrix metalloproteases and neutrophil elastase impede the normal wound healing process. The ECMs are critical for the wound healing process not only because they provide a proper and natural scaffold for cell migration and attachment, but also because they provide a natural reservoir for required growth factors. Studies have suggested that a good collagen-containing biologic therapy can reduce matrix metalloprotease activities by providing
a competitive substrate. Based on the current study, the average quantities of types I, III, and IV collagens, as well as the ratio of types I to III collagen in CSSA, were similar to those in the fresh skin control. The rich and naturally occurring ECM environment could provide an ideal substrate for proteases in wound fluid, as well as spare the degradation of the host tissue. This proper and natural collagen content may tip the balance from development of ongoing necrotic and inflammatory processes in the chronic wound toward a state favoring the desired remodeling and healing processes.

Vascular endothelial growth factor, FGF, and TGF-β are among the most potent angiogenic factors in wound healing. The VEGF is expressed at low levels in normal human skin, whereas its expression is highly unregulated in keratinocytes during wound healing. Both FGF-1 and FGF-2 promote endothelial cell proliferation and differentiation, whereas TGF-β regulates cell proliferation, migration, capillary tubule formation, and ECM deposition. In the current study, comparable amounts of VEGF, IGF-1, FGF-2, and TGF-β were found in CSSA and fresh skin control. Insulinlike growth factor 1, FGF-2, and TGF-β1 were found in the highest quantities in both CSSA and fresh skin.

Allograft skin has a fully developed and mature ECM composition relative to the immature state of newly formed bioengineered tissue products. The benefits of a fully developed ECM found in split-thickness skin should not be underestimated. It is critical that the device or tissue implant has a substantial, well-developed, and natural ECM in order to provide adequate and
optimum collagens and growth factors necessary for healing. The more robust and substantial nature of the allograft ECM also makes it possible to use these products in locations where more fragile bioengineered materials could not be readily used.

The term apoptosis first appeared in the peer-reviewed literature in 1972 to describe a structurally distinct mode of programmed cell death within living tissue. The hallmark changes that an apoptotic cell undergoes are chromatin condensation, DNA laddering, cell shrinkage, plasma membrane blebbing, and fragmentation into compact membrane-enclosed structures, called apoptotic bodies. Apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response. In contrast, during the necrotic mode of cell death, the cellular contents are released uncontrollably into the extracellular environment, which results in damage to surrounding cells and ECM and a strong inflammatory response in the corresponding tissue, which further propagates the cycle of necrotic tissue death in chronic wounds.

The current data demonstrated that tissue recovered within 6 hours of asystole has a low percentage of apoptotic cells in both fresh skin (3.1%) and CSSA (9%). However, because of the small sample size, tissue from more donors will be needed to draw definitive conclusions regarding these observations. Ideally, samples would be taken from the same donor over time and analyzed to better understand the effect of donor variation and WIT on apoptosis. Even though the average percentage of apoptotic cells in CSSA is higher than that in fresh skin, these apoptotic cells are unlikely to cause any inflammatory response in a treated wound, because apoptosis is a necessary part of normal cell life and death. These cells are likely to be phagocytosed by macrophages after the product is placed on the surface of the wound bed.

Cell viability can be tested using various methods, including vital dye staining, oxygen consumption, and enzymatic and metabolic assays. The alamarBlue assay is a well-known cell viability and metabolic assay. Similar to results from other studies, a large variation in cell viability among donors was found in both fresh skin and CSSA. This observation could also be due to the variation in recovery time, especially the WIT, as found in the percentage of apoptotic cell results shown previously. As expected, more viable cells were found in the unprocessed fresh skin than in CSSA; however, the percentage of viable cells maintained in CSSA is consistent with other published data. Many factors can influence cell viability in cryopreserved skin, including warm and cold ischemic time, donor age, transport solution used, and methods of cryopreservation. Because of the inherent donor variation, it is difficult to distinguish the relative contribution of each of these factors on the cell viability of cryopreserved skin, unless samples from a large donor pool are analyzed. In addition, each manufacturer utilizes unique processes to produce cryopreserved split-thickness human skin allograft; the results described here may not be translatable to cryopreserved skin processes using other methods.

CONCLUSIONS

Based on the data presented in this study, CSSA provides a substantial quantity of types I and III collagens, a comparable amount of native growth factors, and the viable cells necessary to be an ideal advanced biologic therapy for repairing chronic wounds in a clinical setting. Donated viable cells and ECM can both provide growth factors that have been shown to help stimulate wound closure.

The current study demonstrated that viable cells are able to survive the process of harvest, cryopreservation, and warming. As a result, the CSSA can be prepared in advance and shipped to a clinical setting, where it can then be applied to a wound to provide growth factors and collagen.

A previous study demonstrated that a CSSA results in rapid closure of wounds with fewer graft applications for both diabetic foot ulcers and venous leg ulcers. Head-to-head clinical studies demonstrated higher closure rates and the need for fewer grafts when comparing CSSA to bioengineered skin substitute. That study attributed the success of CSSA to a more robust and fully developed ECM.

In the current study, the authors demonstrated that a substantial percentage of viable cells survive the harvest, preservation, and warming process necessary to deliver a CSSA to a wound. This allows the CSSA to be an excellent source of collagen and growth factors that are essential to wound healing. Clinical studies cited here support the notion that CSSAs are safe and highly effective and should be considered when the need for an advanced biologic therapy occurs as part of a comprehensive wound care protocol.

REFERENCES


