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Reduced Engraftment and Wound Closure of Cryopreserved Cultured Skin Substitutes Grafted to Athymic Mice

M. Dana Harriger,*,† Andrew P. Supp,* Viki B. Swope,† and Steven T. Boyce*,†

**Shriners Burns Institute and †Department of Surgery, University of Cincinnati, Cincinnati, Ohio, U.S.A.*

Cryopreservation of cultured skin substitutes is a requirement for establishment of banks of alternative materials for treatment of acute and chronic skin wounds. To determine whether cryopreservation of skin substitutes that contain cultured cells reduces their efficacy for wound closure, cell–biopolymer grafts were frozen, recovered into culture, and grafted to wounds on athymic mice. Grafts consisted of cultured human keratinocytes and fibroblasts attached to collagen–glycosaminoglycan substrates that were frozen in cell culture medium with 20% serum and 10% DMSO at a controlled rate and stored overnight in liquid nitrogen. After recovery into culture for 24 h, frozen or unfrozen (control) skin substitutes were grafted to full-thickness wounds on athymic mice. Wound area and surface electrical capacitance were measured at 2, 3, and 4 weeks after grafting at which time animals were sacrificed. Wounds were scored for presence of human cells by direct immunofluorescence staining with a monoclonal antibody to HLA-ABC. The data demonstrate that cell–biopolymer grafts are less efficacious after controlled-rate cryopreservation using 10% DMSO as a cryoprotectant. Frozen grafts at 4 weeks after surgery have significantly smaller wound areas, higher capacitance (wetter surface), and fewer healed wounds that contain human cells. The results suggest that these conditions for cryopreservation of cultured grafts reduce graft viability. Improved conditions for cryopreservation are required to maintain viability and efficacy of cultured skin substitutes after frozen storage.

Rapid coverage and epithelial closure of skin wounds remain major requirements for patient recovery. Inadequate availability of skin autograft and the prospective advantages of reduced numbers of surgical procedures has led to the development of alternative materials for the permanent coverage of acute and chronic skin wounds (20, 23, 25, 28, 30, 33–35). Although many skin substitutes have been described, permanent regeneration of both epithelial and connective tissues after a single surgical application of a skin substitute has not become routine. Permanent closure of acute (10, 11, 30, 31) and chronic (9) wounds has been previously reported using cultured skin substitutes (CSS) consisting of cultured autologous keratinocytes and fibroblasts attached to collagen–glycosaminoglycan substrates. These grafts become fully integrated with fibrovascular tissue and develop functional properties similar to the adjacent, uninjured skin.

A primary goal in tissue banking is to provide a continuous supply of human tissue of the highest quality by preserving the properties critical to engraftment and persistence. The introduction of controlled-rate freezing in conjunction with rapid rewarming has permitted the prolonged storage of viable tissue. Viability of cadaveric skin has been demonstrated to depend on both the rate of rewarming and the limiting warming temperature (42). Despite the common use of cryopreserved skin, current cryopreservation techniques are damaging to epithelial cells, and thus the protective function of the epithelial barrier can be impaired or lost. To facilitate routine use of alternative materials such as the CSS for treatment of acute and chronic skin wounds, development of cryopreservation techniques which yield optimal viability is highly advantageous.

The present study evaluates the effect of cryopreservation on the efficacy of CSS for wound closure after grafting to athymic mice.

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Evaluations include biophysical measurements by surface electrical capacitance (16, 26, 48), wound area, and scoring for presence of human cells by staining for HLA-ABC antigens. Requirements that may be necessary for engraftment and survival of cryopreserved CSS include retention of cellular metabolism and normal tissue anatomy. Therefore, evaluations of CSS were performed before and after cryopreservation by microscopic analysis and a colorimetric viability assay based on the reduction of the tetrazolium salt MTT (44). Understanding the limitations of current cryopreservation methods will permit development of new conditions for cryopreservation that maintain viability and efficacy of CSS after frozen storage. Retention of viability in cryopreserved CSS may provide a continuous supply of the highest quality of skin substitutes for treatment and closure of acute and chronic wounds.

METHODS

Preparation, cryopreservation, and grafting of skin substitutes. Human cells used for the preparation of skin substitutes were derived from donor skin from the Ohio Valley Tissue and Skin Center (Cincinnati, OH, U.S.A.) Skin used to initiate cultures of cells meet the same safety criteria (negative for viral pathogens) that are used for transplantation of cadaver skin.

Primary cultures of human keratinocytes (HK) and human fibroblasts (HF) were established directly from the freshly obtained cadaveric skin using standard methods (12, 13). Primary cultures were harvested by trypsinization after 7–10 days and used to prepare subcultures to greatly expand cell number. Very large populations of HK and HF were prepared, cryopreserved by controlled-rate freezing, stored in liquid nitrogen, and recovered into culture with minimal loss of cell viability. Routine techniques were used to assure maximal cell viability.

Cultured skin substitutes (50 cm^2 each) were prepared as described in previous studies (8, 11, 14) from collagen–glycosaminoglycan (C-GAG) substrates populated sequentially with human dermal fibroblasts and epidermal keratinocytes. Cells were recovered from storage in liquid nitrogen, propagated in culture for 5–7 days, harvested by trypsinization, and then inoculated at 1.0×10^6 cells/cm^2 onto C-GAG substrates. Beginning with inoculation of keratinocytes as Day 0 of incubation, cultured cell–biopolymer composites were incubated 2 days submerged in MCDB 153 medium (12) containing 0.2 m*M* calcium. On Culture Day 2, medium was changed to replace BPE with a lipid supplement (linoleic acid, palmitic acid, oleic acid, arachidonic acid, α-tocopherol) plus carnitine, increased serine (17), and calcium (0.5 m*M*). On Culture Day 3, CSS were lifted to the air–liquid interface on cotton filter pads supported by stainless-steel mesh. EGF was eliminated from the culture medium, and calcium was increased to 1.0 m*M*. On Culture Day 4, calcium was increased to 1.5 m*M*, and CSS were incubated under these conditions with daily medium changes. On Culture Day 13, four CSS were bisected, with half of each prepared for cryopreservation and the parallel halves maintained in culture.

The lipid-enriched medium was supplemented with 10% dimethyl sulfoxide and 20% fetal bovine serum to constitute a cryopreservation medium. The CSS were incubated for 15 min at 4°C in cryopreservation medium, packed flat in sterile tissue pouches containing 15 mL of cryopreservation medium, and subjected to controlled-rate freezing at 1°C/min (21) with a CryoMed Model 1010 microprocessor controlled-rate freezer. Skin substitutes were frozen with a program for cultured cells as summarized in Table 1. Grafts were cooled at 1°C/min to −4°C, and then cooled at 20°C/min through the phase transition during which time the latent heat of fusion was released in a few seconds. After the phase change, the skin substitutes were cooled at 1°C/min to −40°C and then 10°C/min to −90°C at which point the controlled-rate freezing program ended. Frozen packages were transferred, into the vapor phase of liquid nitrogen (circa

TABLE 1
Program of Controlled-Rate Freezing for Cultured Skin Substitutes

Program step	Sample start temp.	Sample end temp.	Cooling rate
Starting temp.	+22°C	+4°C	Ambient on ice
Liquid cooling phase	+4°C	−4°C	1°C/min
Supercooling	−4°C	−4°C	20°C/min
Phase change	−4°C	−4°C	10°C/min
Solid phase I	−4°C	−40°C	1°C/min
Solid phase II	−40°C	−90°C	10°C/min
End solid phase II	−90°C	−90°C	Hold
Liquid N_2 storage	−90°C	−196°C	Vapor phase

−196°C) to simulate long-term storage. One day later, the cryopreserved CSS were recovered into cell culture by rapid warming of the sealed flat pack in a water bath at 37°C. As soon as the skin substitutes were completely thawed (3–4 min), they were rinsed 2 × 15 min in supplemented medium to remove the majority of the DMSO and incubated in air-exposed culture. Skin substitutes were incubated for 1 additional day and cut into 2 × 2-cm pieces for grafting to athymic mice.

All animal studies were approved by the University of Cincinnati, Institutional Animal Care and Use Committee. On Culture Day 14, CSS (n = 12/condition) were grafted to 2 × 2-cm full-thickness wounds in athymic mice as described in previous studies (14–16). The entire wound area was covered with a semipermeable adhesive film (OpSite; Smith & Nephew United; Largo, FL, U.S.A.). This produced a liquid-tight compartment over the wound into which 1.5 cc of sterile keratinocyte growth medium containing antimicrobials (20 μg/mL Ciprofloxacin and 100 U/ml nystatin) was injected through the OpSite into the cotton gauze immediately following surgery. Irrigation medium contained 1.5 m*M* calcium, 5.0 μg/mL insulin, and 0.5 μg/mL hydrocortisone. Dressed grafts were then covered with a self-adherent bandage (Coban; 3M Medical Division, St. Paul, MN, U.S.A.) to protect treated sites from mechanical disturbance. Dressings covering treated sites were injected with 1.0 cc/day of irrigant described above for 9 days after surgery. On Day 10 dressings and stent sutures were removed from all animals, and mice were rebandaged. Dressings were changed, and data were collected on Days 14 and 21 after surgery. On Day 28 after grafting, data were collected, animals were sacrificed, and tissue samples were obtained for immunohistochemistry.

Measurement of surface electrical capacitance (SEC). Hydration of the skin surface is directly proportional to retention of electrical charge or capacitance (16, 45). Measurements of SEC were collected with an impedance based NOVA dermal phase meter (DPM 9003, NOVA Technology Corporation, Gloucester, MA, U.S.A.) connected to a portable computer which recorded 10 serial readings at 1-s intervals.

Data collection and analysis. SEC readings were recorded at 1-s intervals during 10 s of occluded sampling by direct contact of the probe to the CSS surface. Triplicate SEC values were collected from healing CSS grafts on each animal at 2, 3, and 4 weeks after surgery. This provided 36 values/condition/time point. Readings were converted to picoFarads using a standard curve supplied by the manufacturer (16, 24, 48). Data are expressed as means ± SEM in picoFarads. The initial reading (t = 1 s) is defined as instantaneous capacitance. Values recorded after 10 s of occlusion by the probe are defined as continuous capacitance (t = 10 s). Data were subjected to repeated measures analysis of variance ($P < 0.001$) and Tukey's studentized range test with significance accepted at the 95% confi-

dence level ($P < 0.05$). For comparative analysis, SEC data were collected from native human skin using the same method described above for CSS. Triplicate readings were collected from the volar forearm of healthy volunteers ($n = 10$) to yield 30 values.

Wound area tracings and photographs were recorded at Weeks 2, 3, and 4 after grafting, to monitor wound area and engraftment of human keratinocytes. Wound area was determined by direct image analysis (Image-1; Universal Imaging Corporation, Media, PA, U.S.A.) of areas within tracings of wound perimeters. Data for wound contraction are expressed as a percentage of original wound area (mean ± SEM). Data from multiple test groups in wound contraction studies were subjected to analysis of variance.

CSS engraftment was determined by direct immunofluorescence staining of healed epidermis with fluorescein-labeled monoclonal antibody against a common hapten of the HLA-ABC histocompatibility antigens (11, 15, 18). Data for CSS engraftment are expressed as a percentage of HLA-ABC positive wounds in each test condition.

MTT viability assay. To determine the viability of CSS before and after cryopreservation, two parallel CSS were incubated in culture for 14 days, one was maintained in culture and the other was prepared for cryopreservation as above. Twenty-four hours later, the cryopreserved CSS was quickly thawed and six 6-mm punch biopsies were collected from each CSS and prepared for viability assays. Aerobic metabolic activity was determined using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (44). Tetrazolium salts are reduced by dehydrogenase enzymes in metabolically active mitochondria of living cells. A colorimetric assay based on this reduction of MTT was performed. A 24-well plate was prepared with 1.0 mL of MTT solution (0.5 mg/ml in PBS) added to each well. The biopsies were placed in the wells and incubated for 3 h at 37°C. Samples were then transferred to 1.0 ml of 2-methoxyethanol and agitated for 3 h on a rocking platform at room temperature to extract the formazan chromogen which was quantified spectrophotometrically at 590 nm.

Microscopy. Light microscopy was performed by embedment of tissue in glycol methacrylate and staining with toluidine blue. Transmission electron microscopy was performed by preparation of skin substitutes with 2% paraformaldehyde/2% glutaraldehyde in 0.1 *M* sodium cacodylate buffer, pH 7.3. Samples were postfixed in 1% osmium tetroxide, embedded in Epon–Araldite resin, ultrathin sectioned and poststained with lead citrate and uranyl acetate. TEM was performed on a JEOL 100CX II.

RESULTS

Wound closure in athymic mice. Figure 1 shows the external appearance of representative animals from control and cryopreserved conditions 2 and 4 weeks after surgery. Control CSS are epithelialized completely and hyperkeratotic, indicative of human skin on athymic mice. Cryopreservation of CSS causes graft loss by 2 weeks after surgery, leaving open wounds that closed by secondary healing.

Assessment of epidermal barrier formation in vivo. Control grafts have significantly lower SEC values than cryopreserved grafts at all time points (Fig. 2). Capacitance was collected at weekly intervals beginning 2 weeks after grafting. Instantaneous capacitance ($t = 1$ s) (Fig. 2A) demonstrates that control grafts have reduced surface hydration (lower SEC) compared to cryopreserved grafts, and the control grafts approach values for native human skin (14 ± 3 pF) by 2 weeks. Continuous capacitance ($t = 10$ s) (Fig. 2B) of control CSS drops between 2 and 3 weeks to levels approaching native human skin (32 ± 5 pF) and stabilizes through 4 weeks indicating reformation of functional epidermal barrier. Cryopreserved CSS exhibited significantly higher SEC values than control CSS at all time points.

Wound area. Tracings of wound perimeters from healing CSS at 2, 3, and 4 weeks after

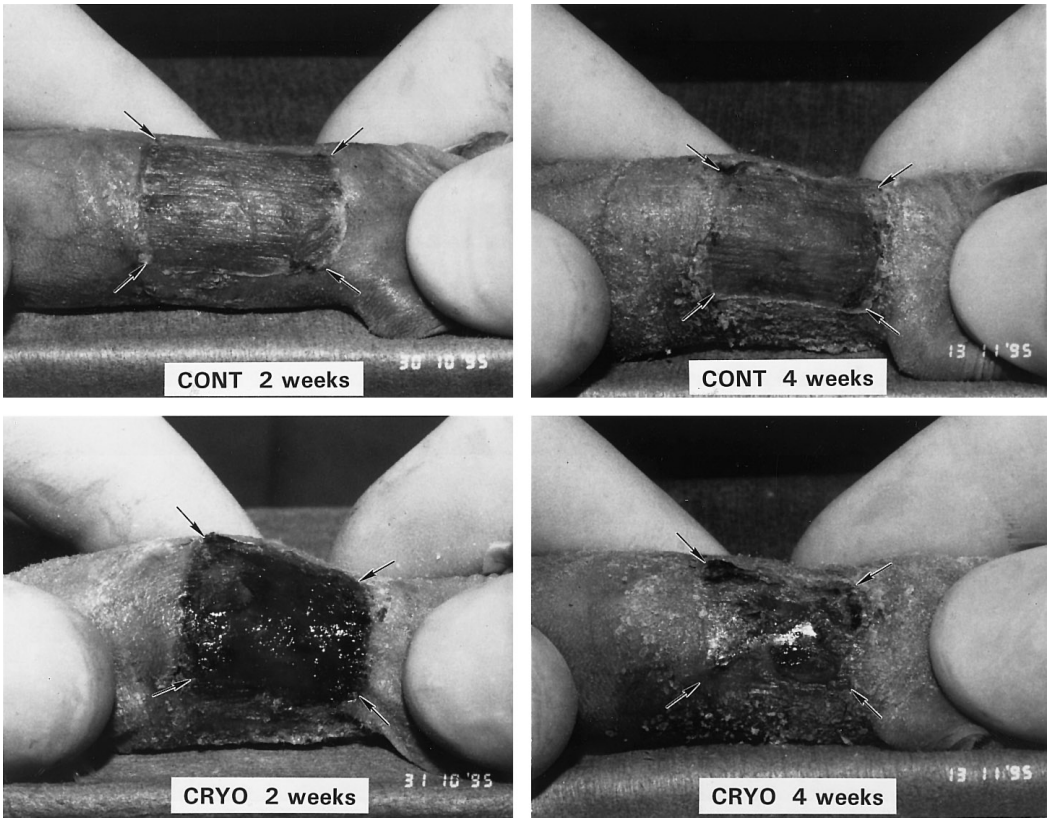

FIG. 1. Photographs of representative animals from control and cryopreserved conditions 2 and 4 weeks after surgery. Control grafts (Cont) are fully fused with wound margins, completely epithelialized and hyperkeratotic by 2 weeks. Cryopreserved grafts (Cryo) are lost at 2 weeks with open wounds beginning to close by secondary healing at 4 weeks.

grafting were converted to area measurements using computerized planimetry. Plots of wound area versus time after grafting (Fig. 3) show wounds treated with control CSS at 3 weeks (57.2 ± 2.9%) and 4 weeks (52.3 ± 3.1%) were significantly larger in percentage of original area than cryopreserved CSS (40.7 ± 2.0% and 33.4 ± 3.4%), respectively.

HLA–ABC expression. Scoring of HLA-ABC expression in cultured grafts at 4 weeks after grafting is shown in Fig. 4. Engraftment occurred in 92% of wounds treated with control CSS compared to 17% of cryopreserved CSS corresponding with the significantly reduced wound area observed.

Histology of CSS before and after cryopreservation. The histologic appearance of CSS prior to grafting to athymic mice is shown in Fig. 5. Human keratinocytes attached to a collagen–glycosaminoglycan substrate populated with human fibroblasts develop a uniformly stratified epithelium that includes cornified layers analogous to stratum corneum. Cryopreserved CSS maintain their epithelial orientation but disruption is evident between the epithelium and the C-GAG substrate (arrows), in addition to vacuolization of epithelial cells as indicated by light microscopy. Transmission electron micrographs confirm cytoplasmic vacuolization and disruption between the basal keratinocytes and the substrate of cryopreserved CSS in comparison to analogous cells in control CSS.

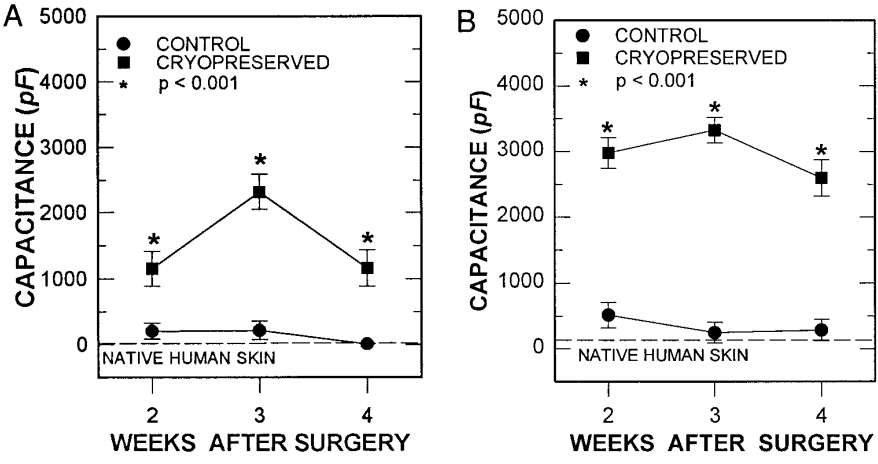

FIG. 2. Assessment by surface electrical capacitance (SEC) of wound hydration for CSS grafted to athymic mice. (A) Plot of instantaneous capacitance (t = 1 s) versus weeks after surgery. Data show that control grafts have reduced surface hydration (lower SEC) compared to cryopreserved grafts, and the control grafts returned to values for native human skin (14 ± 3 pF). Cryopreserved CSS exhibited significantly (*) higher SEC values than control CSS at all time points. Smaller SEM for control CSS indicates more uniform surface hydration. (B) Plot of continuous capacitance (t = 10 s) versus weeks after surgery. Data demonstrate that cryopreserved grafts have significantly (*) higher continuous SEC values than control grafts at all time points. Control CSS approach values for native human skin (32 ± 5 pF) by 3 weeks after surgery, indicating restoration of epidermal barrier function.

MTT viability analysis. Figure 6 shows the plot of the MTT analysis from the control and cryopreserved CSS. Cryopreservation of CSS causes a significant threefold decrease in cell viability from control CSS as measured by MTT reduction.

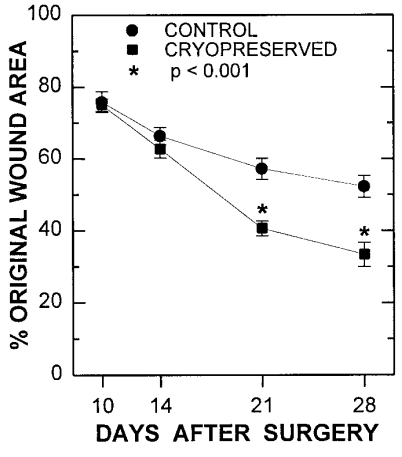

FIG. 3. Plot of percentage original area versus days after surgery (mean ± SEM). Wounds treated with control CSS were significantly (*) larger in area than cryopreserved CSS at 21 and 28 days after surgery.

DISCUSSION

Data presented in this report quantify injury to structure and function of cultured skin substitutes that results from cryopreservation. Previous reports from this laboratory have shown that autologous human keratinocytes and fibroblasts attached to an implantable collagen–glycosaminoglycan substrate allow for rapid attachment to the wound bed while permitting ingrowth of fibrovascular tissue. Histological and ultrastructural analysis of the healed skin after grafting to burn patients reveal attributes of a fully healed functional skin. The data presented here demonstrate that cell–biopolymer grafts are less efficacious after controlled-rate cryopreservation using 10% DMSO as a cryoprotectant.

Possible mechanisms of injury to skin substitutes by cryopreservation include actions of

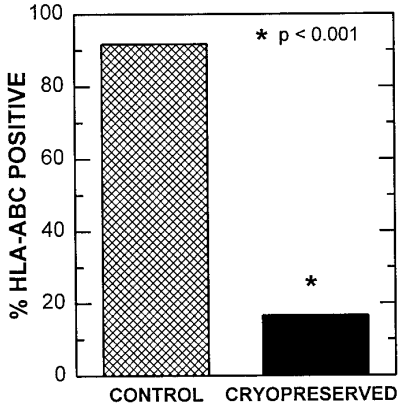

FIG. 4. Plot of percentage HLA-ABC positive wounds versus graft treatment. Engraftment of control CSS occurred in 92% (11 of 12) of the animals as demonstrated by positive expression of HLA-ABC at 4 weeks after grafting. Engraftment with cryopreserved CSS was supported by only 17% (2 of 12) of the animals, showing a significant difference (*) from the control animals.

physical and chemical agents. Ice crystallization is the primary mechanism by which cell damage occurs, and cryoprotectants (i.e., DMSO) reduce physical injury by decreasing formation of crystalline ice. However, the cryoprotectant DMSO may also cause chemical injury. Although a control for DMSO exposure in the absence of freezing was not included in this study, 10% DMSO is used routinely in this laboratory with recovery of 60–75% viability of cultured human cells in suspension (10, 12). Therefore, the probability of cellular injury by exposure to DMSO is not as great as physical damage from crystalline ice. Because skin substitutes have a thickness of approximately 0.3–0.5 mm, two factors may increase ice damage. First, it is possible that DMSO does not penetrate fully into the thickness of the material, but in consideration of the rapid movement of DMSO in biologic tissue, this seems unlikely. Rather, it is more probable that ice formation begins at the surface of the skin substitute and moves inward. If so, then ice formed on the exterior of the material acts to insulate the movement of heat from the interior of the skin substitutes and thereby slows the freezing rate. Introduction of an insulating material at the surface of the graft may generate a gradient of freezing rate from exterior to interior that may result in greater crystallization of ice inside the material and reduced viability consequently. This kind of mechanism may also account for decrease of viability in cryopreserved cadaveric skin in tissue banking.

Viable human skin from cadaveric sources has proven to be a very effective biologic dressing to cover excised, deep second- or third-degree burns if sufficient amounts of autograft skin are not available (1, 2, 19, 22, 27, 36, 40, 41). Application of the principle of early burn excision and grafting (37), generates a demand for optimal and rapid wound coverage. Split-thickness cadaveric allograft skin remains the treatment of choice to cover excised burns if either the extent of the burn area or the patient's condition does not allow early completion of wound coverage with autograft skin. Despite the established usefulness of allograft skin in burn wound management, the importance of allograft viability on successful outcome remains virtually undetermined. The debate over the advantages of fresh versus preserved allograft continues today (3, 6, 38, 46). Although selected studies report the successful application of nonviable cryopreserved skin (4, 32, 39), there is a consensus that the more viable the allograft, the better its performance as a temporary wound coverage in patients with large thermal injuries (7, 43, 47).

To introduce the routine use of alternative treatments for skin wounds, cryopreservation and recovery of viable skin composites is directly related to availability. Cryopreservation procedures used in this study follow generally those currently accepted by tissue banks for human allograft (38). Histologic differences were apparent between cryopreserved and control grafts *in vitro.* Upon removal of wet dressings at 10 days after surgery, distinct differences were observed between the two conditions. Wounds treated with cryopreserved CSS had poorly adhered grafts or open wounds which healed by secondary intention

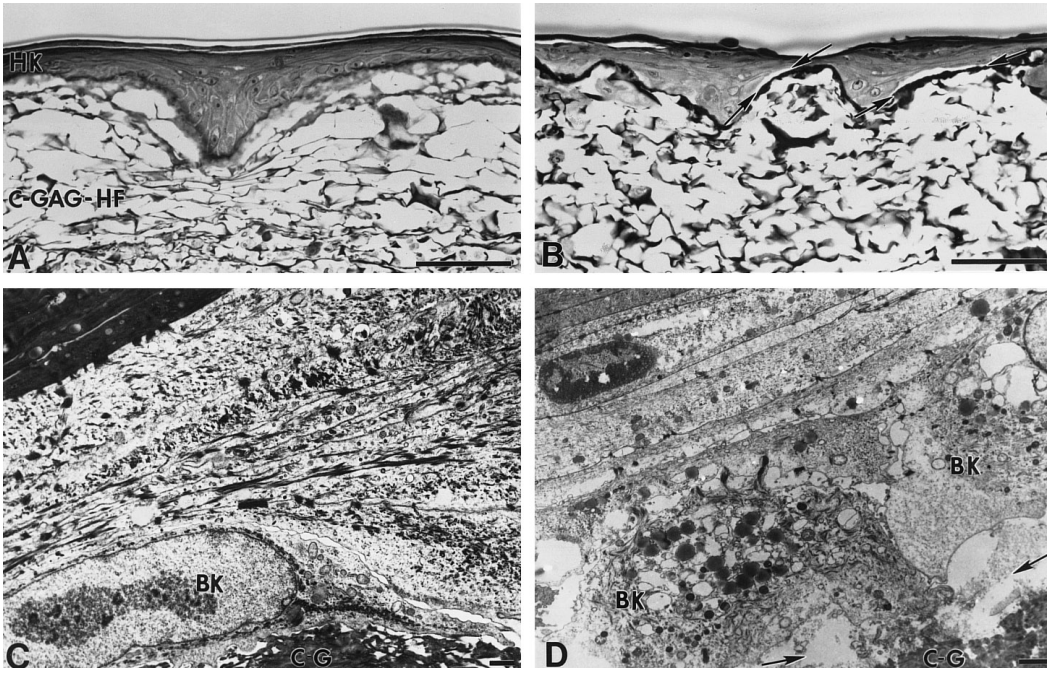

FIG. 5. Micrographs of cultured skin substitutes before and after cryopreservation. (A) By Day 14 *in vitro*, a uniformly stratified epithelium of human keratinocytes (HK) develops on a collagen–glycosaminoglycan substrate populated with human fibroblasts (C-GAG-HF). (B) Cryopreserved CSS maintain their epithelial orientation but separation from the C-GAG substrate (arrows) is apparent. Ultrastructural analysis reveals cytoplasmic vacuolization and separation of the basal keratinocytes (BK) from the underlying collagen–glycosaminoglycan (C-G) matrix of cryopreserved CSS (arrows) (D) in comparison to analogous cells in control CSS (C). Scale bars: light micrographs, 100 μm; electron micrographs, 1 μm.

within 4 weeks. Wounds contracted down to 33.4 $\pm$ 3.4% of the original wound area. This was consistent with the HLA immunostaining that showed that only 17% of the cryopreserved grafts were retained compared to 92% of the control grafts. Surface electrical capacitance demonstrated significantly higher values at all weekly time points for cryopreserved grafts indicating failure of barrier function of the epithelium.

Comparison of cryopreserved CSS to control CSS in this study has exhibited a reduction in adherence to the wound after grafting to athymic mice leading to healing by secondary intention and increased wound contraction. Cryopreserved allograft has been reported to undergo early separation of the injured epidermal layer following placement of the skin on the wound (29). Although there are limitations when using cryopreserved allograft, many burn centers must rely on cryopreserved skin because a sufficient supply of ''fresh'' allograft is not available. Therefore, development of cryopreservation techniques which yield optimal skin viability has clear medical advantages. The primary purpose in tissue banking is to provide a continuous supply of the highest quality of allograft skin by preserving the tissue properties required for graft take and survival. To achieve this goal, viability assays are used to measure cellular injury for development of protocols that minimize damage and allow quality assurance of cryopreserved skin. Requirements that may be necessary for the engraftment and survival of skin are retention of living, functioning cells and a normal microscopic and macroscopic dermal structure (42, 46). These requirements must also apply

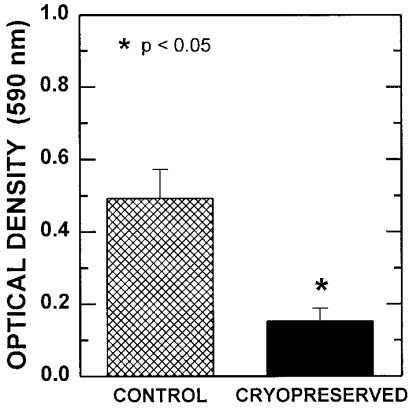

FIG. 6. Plot of optical density values from an MTT colorimetric assay on CSS before and after cryopreservation. Cells in the control CSS reduce more MTT to formazan than those in the cryopreserved CSS indicating a significant threefold decrease in metabolic viability after cryopreservation.

to alternative materials if they are to be validated for healing of skin wounds. Cryopreservation conditions will be optimized by modification of formulae of cryopreservation solutions (i.e., cryoprotectant and protein concentrations) and by modification of freezing rates for minimal freeze damage to skin substitutes.

The findings of this report provide additional evidence to suggest that composite cultured skin substitutes form a functional full-thickness skin after grafting to athymic mice. Improvement of protocols for cryopreservation will ultimately facilitate long-term storage of a viable, ready-to-use skin substitute for treatment and closure of skin wounds.

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