

Comparison of Therapeutic Antibiotic Treatments on Tissue-Engineered Human Skin Substitutes

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ABSTRACT

For regenerative medicine to gain clinical acceptance, the effects of commonly used treatment regimens on bioengineered organs must be considered. The antibiotics mafenide acetate (mafenide) and neomycin plus polymyxin (neo/poly) are routinely used to irrigate postoperative skin grafts on contaminated wounds. The effects of these clinically used antibiotics were investigated using tissue-engineered human skin substitutes generated with primary human keratinocytes or the near-diploid human keratinocyte cell line, Near-diploid Immortal Keratinocytes. Following topical or dermal treatment, the skin substitutes were assayed for viability, tissue morphology, glycogen content, and the expression of active caspase 3. Mafenide, but not neo/poly, induced morphological and biochemical changes in tissue-engineered skin substitutes. Keratinocytes in all histological layers of mafenide-treated skin substitutes exhibited ballooning degeneration and glycogen depletion. Mafenide-treatment also triggered separation of basal keratinocytes from the underlying dermis. None of the antibiotic treatments induced apoptosis, as measured by active caspase 3 immunostaining. The results demonstrate that mafenide, but not neo/poly, is detrimental to the viability and structural integrity of tissue-engineered human skin substitutes. These findings highlight the need to identify treatment regimens that are compatible with and hence enable the therapeutic efficacy of first-generation bioengineered organs such as skin.

INTRODUCTION

CELL SOURCING FOR TISSUE ENGINEERING is a well-recognized challenge for the field of regenerative medicine.¹ Human skin was the first human organ to be successfully bioengineered. The first generation of tissue-engineered human skin substitutes have relied on cells sourced from multiple screened donors or from autologous cells.² Human skin is a renewal tissue with epidermal stem cells residing in the hair follicle and interfollicular epidermis.^{3,4} The presence of epidermal stem cells and progenitors is key to the success of tissue engineering efforts for human skin substitutes. The novel human keratinocyte

progenitor cell line, NIKS (Near-diploid Immortal Keratinocytes), exhibits normal keratinocyte differentiation characteristics and an extended lifespan *in vitro*. These near-diploid cells were derived from a population of diploid, primary human keratinocytes. Our laboratory has previously demonstrated that, unlike other immortalized keratinocyte cell lines, NIKS retains cell type-specific growth requirements and differentiation properties, is non-tumorigenic, is virus free, and recapitulates full skin architecture in organotypic culture.⁵ The NIKS cells are a consistent source of long-lived, pathogen-free, allogenic, human epidermal progenitor cells, and are an attractive candidate for therapeutic clinical applications. Recently,

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tissue-engineered human skin substitutes generated with NIKS keratinocytes have gained clearance for clinical investigation in a phase I/phase II clinical trial focused on treatment of complex skin defects. Severe trauma such as degloving injuries, third-degree burns, and chronic non-healing skin ulcers are all examples of skin defects that represent significant challenges in wound closure and tissue regeneration.

Infection remains a major obstacle in wound closure, despite the many advances in wound care and tissue engineering.⁶⁻⁸ According to the American Burn Association Burn Registry data from 1974 to 2005, wound infection was among the top five most common complications for patients admitted with burn injury.⁹ Clinical experience has shown that mortality is lowered with early excision and grafting.¹⁰ In situations where this standard of care is not feasible as in cases of large total body surface area (TBSA) defects or poor intrinsic wound healing, wound closure may be delayed or necessitate the use of human skin substitutes containing keratinocytes grown on collagen embedded with fibroblasts. In comparison to autografts, human skin substitutes require more time for vasculogenesis and engraftment and are at an increased risk of graft failure due to infection.¹¹ For these reasons, postoperative irrigation of the skin graft with antimicrobials is desirable. Additionally, microorganisms resistant to common antimicrobials, such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Methicillin-resistant *Staphylococcus aureus* (MRSA), pose immense challenges in the treatment of wound infections and require constant reevaluation of antimicrobial treatment regimens.

Therapeutic and prophylactic use of antibiotics is common in the treatment of complex skin defects. In patients with contaminated wounds, split-thickness meshed autografts are often irrigated postoperatively with alternating solutions of mafenide acetate (mafenide) and neomycin plus polymyxin (neo/poly). These solutions are instilled through red-rubber catheters within the dressing every 2 h to provide topical antimicrobial prophylaxis and a moist wound environment. Given the typical bacterial species prevalent in burn wounds, neo/poly and mafenide solutions are an attractive therapy due to the wide spectrum of activity against gram-positive and gram-negative bacteria.^{12,13} Like split-thickness autografts, NIKS human skin substitute tissue can be readily meshed; therefore, both the epidermal and dermal surface of the bio-engineered tissue would be exposed to therapeutic doses of commonly used antibiotic solutions in a clinical setting. To date, there are no reports on the morphologic and metabolic effects of these antibiotics on tissue-engineered human skin substitutes. In this study, we investigated the effects of epidermal and dermal exposure of two commonly used antibiotic solutions, neo/poly and mafenide, on the viability and tissue morphology of human skin substitutes generated using primary human keratinocytes or the NIKS epidermal progenitor cell line.

MATERIALS AND METHODS

Monolayer cell culture methods

Primary human epidermal keratinocytes were isolated from newborn foreskins. Samples were obtained after circumcision under the approval of both Madison Meriter Hospital and the University of Wisconsin Human Subjects Committees and Institutional Review Boards. Primary human keratinocyte cultures were established by plating aliquots of a single-cell suspension in the presence of mitomycin C-treated Swiss mouse 3T3 fibroblasts (mito-3T3) as previously described.¹⁴ Both primary human keratinocytes and NIKS keratinocytes⁵ were cultivated in standard keratinocyte culture medium composed of a mixture of Ham's F-12 medium:Dulbecco's modified Eagle's medium, (3:1, final calcium concentration 0.66 mM) supplemented with 2.5% Fetal Clone II (Hyclone, Logan, UT), 0.4 µg/mL hydrocortisone, 8.4 ng/mL cholera toxin, 5 µg/mL insulin, 24 µg/mL adenine, 10 ng/mL epidermal growth factor, and 100 units penicillin. Both primary human keratinocytes and NIKS keratinocytes were subcultured at weekly intervals at 3×10^5 cells per 100-mm tissue culture dish (approximately a 1:25 split) onto a mito-3T3 feeder layer.

Antibiotic treatment of NIKS and primary human keratinocytes in monolayer culture

NIKS and primary human keratinocytes were each seeded to six-well plates at a density of 5×10^4 cells/well onto a mito-3T3 feeder layer in antibiotic-free standard keratinocyte medium. When cultures reached confluence, they were fed standard keratinocyte growth medium with or without the indicated antibiotics every other day for 7 days. Neo/poly (Neosporin G.U. Irrigant; Monarch Pharmaceuticals, Bristol, TN) treatments of 40 µg mL⁻¹/200 units mL⁻¹, 20 µg mL⁻¹/100 units mL⁻¹, 4 µg mL⁻¹/20 units mL⁻¹, 2 µg mL⁻¹/10 units mL⁻¹, and 0.4 µg mL⁻¹/2 units mL⁻¹ were administered as indicated. Mafenide (Sulfamylon; Bertek Pharmaceuticals, Sugar Land, TX) treatments of 50, 5.0, 2.5, 0.5, 0.25, and 0.05 mg/mL were administered as indicated. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to assess cell viability (see MTT method below). Treatments were compared to the untreated control consisting of standard keratinocyte medium without antibiotics. Triplicate samples were assayed, and at least three experiments were conducted.

Organotypic cell culture methods

Composite human skin substitutes were prepared using NIKS or primary human keratinocytes and human fibroblasts. Skin substitutes were generated by organotypic culture of keratinocytes on an HA (mixed cellulose esters)-membrane Millicell culturing system (Millipore, Billerica, MA). Briefly, normal human fibroblasts at a density of 3.6×10^4 cells/well

were cultured with type 1 collagen for 4 days to form a cellularized dermal matrix. NIKS or primary human keratinocytes were seeded onto the surface of the cellularized dermal matrix at a density of 3.75×10^5 cells/well and maintained at the air/medium interface throughout the stratification process. Full stratification is typically present by day 14 of organotypic culture.⁵

Dermal antibiotic treatment of human skin substitute

Human skin substitutes were used at days 17 and 18, and exhibited full stratification at the time of antibiotic treatment. Tissues were treated in 1.8 mL of StrataLife™ culture medium (Stratatech, Madison, WI) containing either: mafenide (50 mg/mL); neomycin sulfate (40 µg/mL) and polymyxin-B sulfate (200 units/mL); mafenide plus neo/poly; or media only placed in contact with the dermal component of the tissue. Medium, with or without these therapeutic concentrations of antibiotics, was replaced daily. At 48 and 96 h after daily antibiotic addition, cell viability was assessed using an MTT assay (Sigma-Aldrich, St. Louis, MO). Triplicate samples were assayed, and at least three experiments were conducted.

Topical antibiotic treatment of human skin substitutes

To mimic the clinical application of these antibiotics on the epidermal surface, human skin substitutes were treated topically with antibiotics. NIKS or primary human keratinocytes were used to generate the skin substitutes as describe above. Therapeutic concentrations of mafenide (50 mg/mL), neomycin sulfate (40 µg/mL) and polymyxin-B sulfate (200 units/mL), or a combination of mafenide plus neo/poly were placed onto the intact human skin substitutes on days 17 and 18 of culturing. For each Milliwell culture, 100 µL of media with or without antibiotics was placed on the epidermal surface of the skin substitutes. Residual media were aspirated from the epidermal surface of the cultures daily prior to replacement of fresh control or antibiotic-containing medium. At 48 and 96 h, viability was assessed using an MTT assay. Triplicate samples were assayed, and at least three experiments were conducted.

MTT assay for cell viability

For skin substitute tissues, medium was removed from antibiotic-treated and control tissues and replaced with 300 µL of medium containing 1 mg/mL of MTT. Tissues were incubated for 3 h at 37°C. The MTT-formazan product was extracted by gently shaking with 2 mL isopropanol overnight. The optical absorbance of the extracts was determined at 540 nm. The viability of each tissue relative to the average of the antibiotic-free control tissues was calculated by dividing the A_{540} of the experimental condition medium by the average of the A_{540} of the control medium.

Results are expressed as percent of control in bar graph format and contain the mean and standard error of the mean.

For keratinocytes grown in monolayer culture, medium was removed from antibiotic-treated and control cultures and replaced with 1 mL of medium containing 1 mg of MTT. Monolayer keratinocytes were incubated for 2 h at 37°C. Media were aspirated, and cells rinsed with phosphate-buffered saline (PBS). The MTT-formazan product was extracted by incubation with 2 mL isopropanol while shaking gently for 2 h. The optical absorbance of the extracts was determined at 540 nm. The viability of the cells relative to the average of antibiotic-free control cultures was calculated by dividing the A_{540} of the experimental condition medium by the average of the A_{540} of the control medium. Results are expressed as percent of control in line graph format and contain the mean and standard error of the mean.

Histological assessment of human skin substitutes

Skin substitute tissues were fixed in 4% paraformaldehyde and embedded in paraffin, and 5 µm sections were prepared. These sections were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) to assess tissue histology and the presence of glycogen, respectively. Amylase digestion was used to confirm specificity of the PAS stain for glycogen. Stained sections were viewed and photographed using an Olympus inverted microscope IX70 (Leeds Precision Instruments, Minneapolis, MN) equipped with a digital camera.

Indirect immunofluorescence detection of active caspase-3

Fixed tissues were embedded in paraffin, and 5 µm sections were prepared. Active caspase 3 was detected in sections by indirect immunofluorescence (IIF) using a purified rabbit antihuman/mouse active caspase 3 antibody (R&D Systems, Minneapolis, MN). Briefly, deparaffinized sections were blocked with 5% normal goat serum in PBS at room temperature for 45 min. Active caspase 3 antibody was diluted (1:50) with 5% normal goat serum in PBS and incubated on tissue sections overnight at 4°C. Tissue sections incubated with 5% normal goat serum in PBS served as controls for nonspecific staining. Goat anti-rabbit Alexa-488 (Molecular Probes, Eugene, OR), diluted 1:500, was used as a secondary antibody. The tissue samples were incubated in the dark for 40 min at room temperature. Nuclei were stained with 33258 Hoechst (5 mg/mL) (Acros, Geel, Belgium). The positive control for apoptosis was generated by treating human skin substitute tissue with 5 µg/mL cyclohexamide for 24 h prior to tissue fixation and indirect immunofluorescence analysis for active caspase 3. Tissue samples were viewed using the Hoechst and fluorescein filters on an Olympus inverted microscope. For each

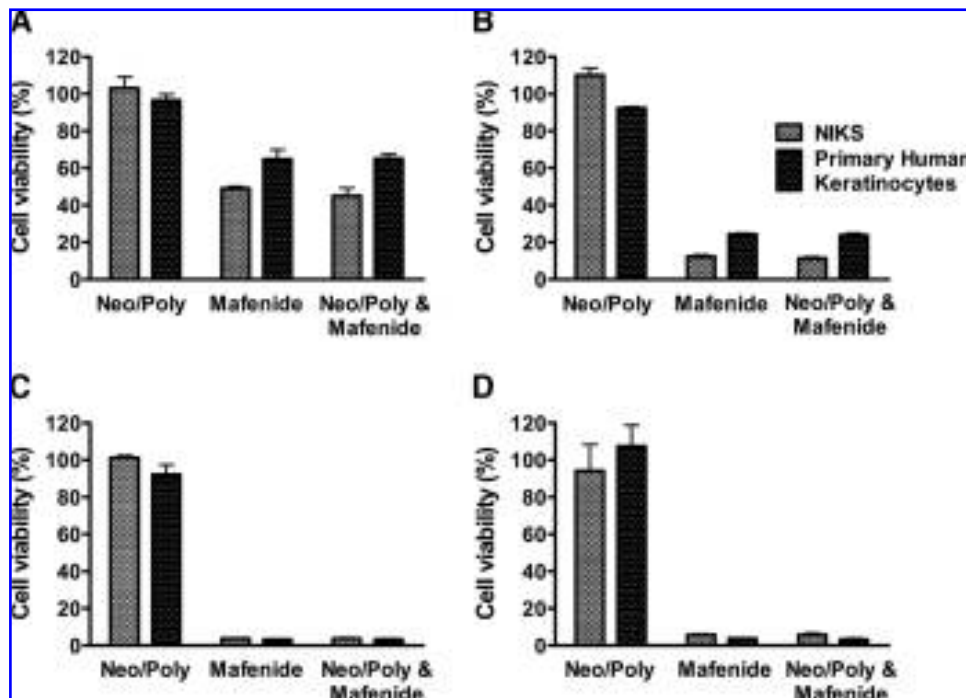


FIG. 1. Tissues were treated daily with the indicated antibiotic topically for 48 h (A) or 96 h (B), or dermally for 48 h (C) or 96 h (D). Viability, as determined by an MTT assay, is expressed as a percentage of the no antibiotic controls. $p < 0.0002$ for neo/poly versus mafenide or neo/poly + mafenide in NIKS and primary human keratinocytes, two-way ANOVA. Data are representative of triplicate experiments.

treatment, active caspase 3–positive cells were counted in duplicate tissue sections of 8 mm in length. At least three experiments were conducted.

Statistical analysis

Viability was computed for each tissue as a percentage of the average untreated control values. The statistical analysis was based on the average viability for each tissue (i.e., average over triplicate). Two-way analysis of variance (ANOVA) models were used to test for differences in viability. The models for the monolayer experiments included main effects for tissue type (NIKS, primary human keratinocytes) and dose, as well as an interaction term; a separate model was fitted for each agent (mafenide, neo/poly). The models for the topical and dermal application experiments included main effects for tissue type and agent (mafenide, neo/poly, neo/poly + mafenide), and their interaction. If significant differences were found for a given effect, pairwise comparisons between least-squares means were examined (Fisher's protected least significant difference test). To protect against inflating the type I error rate, only the following types of contrasts were examined: differences between dose within a given tissue type, differences between tissue within a given dose, and differences between agents. p -values < 0.05 were considered significant. The ANOVA models were fitted with SAS Proc Mixed, version 9.1.3 (SAS Institute, Cary, NC).

RESULTS

Application of mafenide to human skin substitutes results in loss of viability and tissue architecture

Allogenic human skin substitute tissue generated from NIKS keratinocytes can be meshed using standard surgical meshing instruments. In a clinical setting, both the epidermal surface and dermal surface of a meshed tissue-engineered skin substitute would be exposed to therapeutic doses of antibiotic solutions. For this reason, we investigated the effects of antibiotic treatment on the viability of human skin substitutes, exposing the dermal or epidermal surfaces to mafenide or neo/poly individually or in combination. Composite human skin substitutes contained primary human keratinocytes or NIKS keratinocytes in the epidermis and primary dermal fibroblasts in the dermis. Treated tissues were incubated with the antibiotics for 48 or 96 h and assayed for viability. Mafenide, but not neo/poly, caused a dramatic loss of cell viability in the skin substitutes by 48 h (Fig. 1). Treatment of the dermal surface of the skin substitutes with mafenide alone or in combination with neo/poly resulted in a more extensive and rapid loss of cell viability relative to topical treatment of the epidermis. However, by 96 h even topically-treated skin substitutes had 75% fewer viable cells compared to controls. These findings show that exposure of human skin substitutes to mafenide through the dermal surface reduces viability to a greater extent than

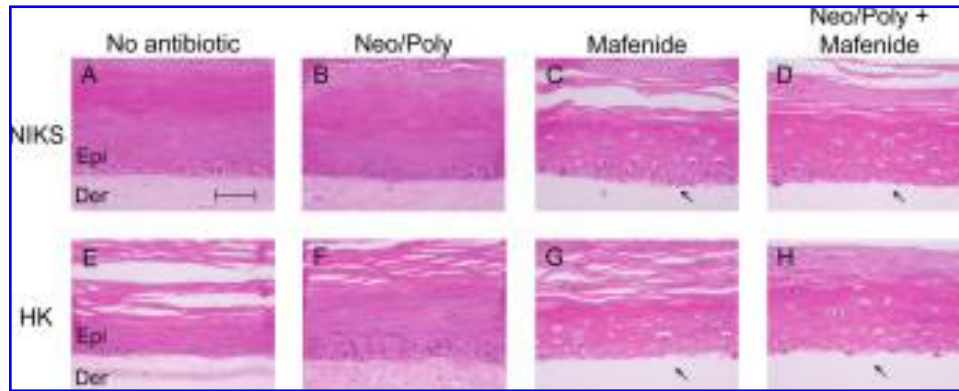


FIG. 2. H&E sections of NIKS (A–D) and primary human keratinocytes (HK) (E–H) after 48 h of dermal surface treatment with neo/poly (B, F), mafenide (C, G), or a combination of neo/poly and mafenide (D, H). Scale bar represents 50 μ m. Arrows indicate the loss of cell–substratum contact. Data are representative of triplicate experiments. Epi, epidermal layer; Der, dermal layer.

does topical exposure. Treatment with neo/poly had a modest effect on the viability of the skin substitutes regardless of the method of exposure. Tissues treated topically with mafenide became more permeable as evidenced by the inability to recover the mafenide-containing solutions from the epidermal surface of the skin substitute. This was in direct contrast to recovery of the full volume of both the neo/poly topical treatment and no antibiotic control topical treatment. These findings suggest that topical mafenide exposure may also compromise the permeability barrier function of human skin substitutes.

Histological analysis of antibiotic-treated human skin substitutes was consistent with the effects of mafenide and neo/poly on tissue viability. Morphological responses were observed in the keratinocytes following treatment of the dermal and epidermal surfaces of the skin substitutes with mafenide but not neo/poly. Changes at 48 h included the formation of perinuclear vacuoles in keratinocytes located in the suprabasal layers of the epidermis, and detachment of basal keratinocytes from the dermis (Fig. 2). However, no histological changes in dermal fibroblasts were observed.

Topical treatment with mafenide for 48 h (Fig. 3) and 96 h (data not shown) resulted in extensive hydropic swelling (ballooning degeneration) of keratinocytes in all histological layers of the epidermis with the exception of the stratum corneum. Neo/poly did not induce detachment of the epidermis from the dermis, hydropic swelling, or perinuclear vacuolization of keratinocytes (Figs. 2 and 3). Further, the cytotoxic and morphological effects of mafenide on human skin substitutes were not affected by cotreatment with neo/poly (Figs. 2 and 3). These studies revealed new mafenide-induced changes in human skin substitutes, including swollen, vacuole-containing keratinocytes in all living layers of the epidermis, delamination of the epidermal compartment, and overall decreased tissue viability.

Mafenide reduces viability of NIKS and primary human keratinocytes in monolayer culture

The skin substitutes used in our studies contain both epidermal keratinocytes and dermal fibroblasts. Formation of a stratified squamous epithelium is dependent on

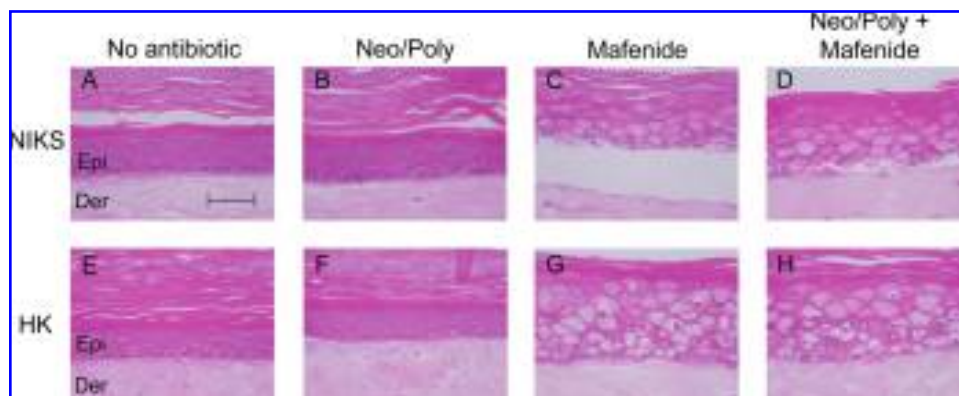


FIG. 3. H&E sections of NIKS (A–D) and primary human keratinocytes (HK) (E–H) after 48 h of topical surface treatment with neo/poly (B, F), mafenide (C, G), or a combination of neo/poly and mafenide (D, H). Scale bar represents 50 μ m. Data are representative of triplicate experiments. Epi, epidermal layer; Der, dermal layer.

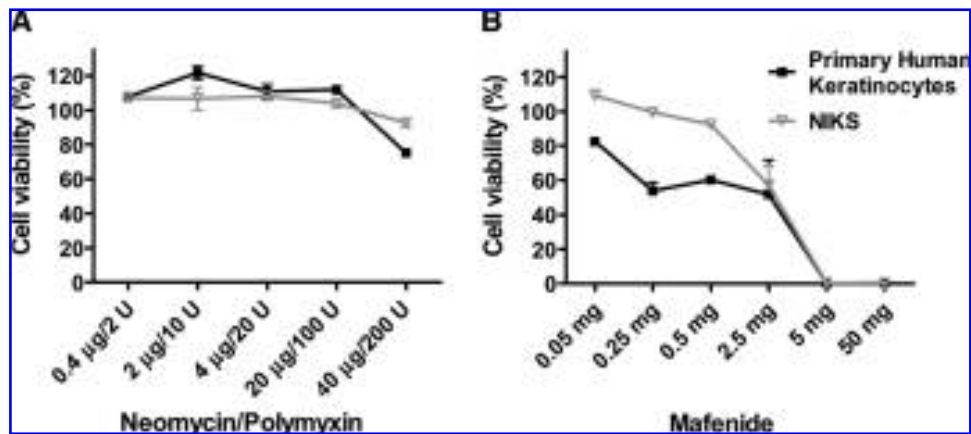


FIG. 4. Confluent monolayer cultures of primary human keratinocytes and NIKS keratinocytes were treated for 7 days with neo/poly (A) or mafenide (B), and cell viability was assessed. Viability, as determined by an MTT assay, is expressed as a percentage of the no antibiotic controls. * $p < 0.05$ for dosages 0.05, 0.25, 0.5, and 2.5 mg versus 50 mg mafenide, two-way ANOVA. Data are representative of triplicate experiments.

dermal–epidermal signaling that supports cell division in the basal layer and cell movement of differentiating keratinocytes into suprabasal layers.¹⁵ To determine the effect of neo/poly and mafenide on keratinocytes alone, we treated confluent monolayer cultures with medium containing various concentrations of neo/poly or mafenide for 7 days. Neo/poly treatment resulted in a modest decrease in viability of both primary human keratinocytes and NIKS keratinocytes that was not dose dependent (Fig. 4A). In contrast, mafenide caused a dramatic dose-dependent decrease in viability of both primary and NIKS keratinocytes (Fig. 4B). Cell viability was reduced by 99% relative to control cultures when monolayer cultures were treated with greater than 5 mg/mL mafenide. Treatment with increasing concentrations of mafenide induced a progressive loss of cell–substratum attachment but not cell–cell attachment, ultimately resulting in a fragmented sheet of detached keratinocytes. The loss of cell–substratum attachment observed in mafenide-treated keratinocyte monolayer cultures mimicked the loss of basal keratinocyte attachment to the dermis found in mafenide-treated human skin substitutes. Neo/poly-treated keratinocytes showed no change in cell–substratum or cell–cell adhesion compared to controls. These studies with monolayer cultures show that mafenide treatment reduces keratinocyte viability and specifically promotes loss of cell–substratum adhesion but not cell–cell adhesion. Overall, mafenide-induced effects on keratinocytes were cell autonomous affecting cell viability and adhesion to substrata independent of the culture format or the presence of dermal fibroblasts.

Human skin substitutes treated with mafenide lack glycogen and active caspase 3

To identify cellular pathways involved in mafenide's effects on tissue-engineered human skin substitutes, we as-

sessed two indicators of cellular stress, glycogen content and caspase 3 status. Stress-induced hydropic swelling and cellular vacuoles are observed in other epithelial cell types, including keratinocytes.^{16,17} In tape-stripped keratinocytes, cellular vacuolization is associated with the accumulation of glycogen and is an adaptive response to acute cellular stress.¹⁶ Keratinocyte apoptosis is also a cellular response to variety of stresses. It is unknown if antimicrobial agents at therapeutic concentrations induce stress responses such as glycogen storage or apoptosis in human skin.

PAS staining was conducted to assess the content and distribution of glycogen-containing cells in human skin substitutes. In NIKS human skin substitutes, glycogen was abundant in keratinocytes located in the spinous and granular layers, but was absent in basal keratinocytes and the corneocytes of the stratum corneum (Fig. 5A, E). This pattern of glycogen distribution in the epidermal compartment of NIKS skin substitutes was also found in primary human keratinocytes skin substitutes (data not shown) and is identical to that reported for the epidermis of intact human skin.¹⁸ Topical or dermal treatment with neo/poly had no effect on the glycogen content or distribution of glycogen-expressing keratinocytes in human skin substitutes (Fig. 5B, F). However, both topical and dermal treatments containing mafenide resulted in a loss of glycogen in keratinocytes located in the spinous and granular layers (Fig. 5C, D, G, H). Human skin substitutes generated with primary human keratinocytes exhibited patterns of glycogen distribution following topical or dermal antibiotic treatments identical to that of NIKS (data not shown). No glycogen staining was observed following amylase digestion of control or antibiotic-treated tissues confirming the specificity of the PAS stain. These studies show that mafenide induces a unique pattern of glycogen depletion in the suprabasal layers of the epidermal compartment of human skin substitutes.

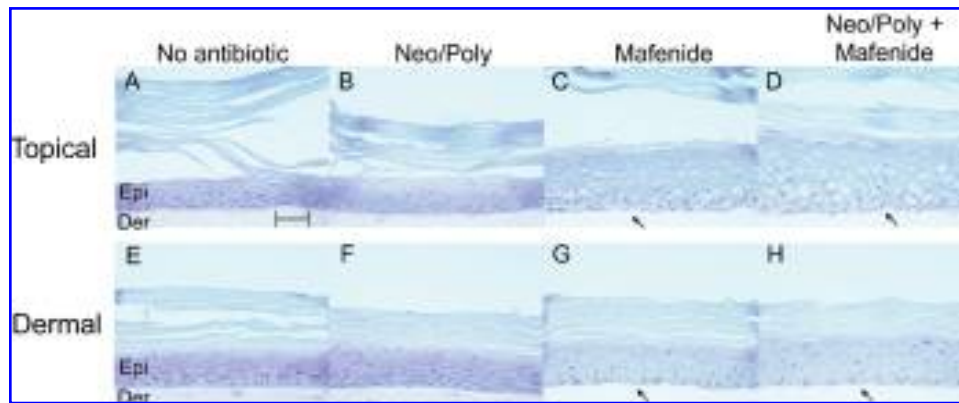


FIG. 5. PAS-stained sections of NIKS keratinocytes after 48 h of topical (A–D) or dermal (E–H) application of neo/poly (B, F), mafenide (C, G), or a combination of neo/poly and mafenide (D, H). Scale bar represents 50 μ m. Staining for glycogen is indicated by purple coloration within the cells. Arrows indicate the loss of cell–substratum contact. Epi, epidermal layer; Der, dermal layer.

Finally, we investigated the ability of antibiotic treatments to induce active caspase 3 in human skin substitutes. Human keratinocytes have been shown to undergo apoptosis through both caspase-dependent and -independent pathways. Active caspase 3 is the primary cell death effector for both extrinsic and intrinsic apoptotic signaling in numerous cell types, including keratinocytes.¹⁹ NIKS and primary human keratinocyte human skin substitutes treated for 48 h with neo/poly or mafenide applied topically or dermally exhibited few active caspase 3–positive keratinocytes all of which were located in the basal layer of the epidermal compartment. Due to the extensive ballooning degeneration of mafenide-treated keratinocytes and epidermal delamination, active caspase 3–positive basal keratinocytes were enumerated in the entire 8 mm length of duplicate histological sections (representative data presented in Table 1). Skin substitute tissues treated with cyclohexamide served as a positive control for active caspase 3 staining and contained an average of 55 positive basal keratinocytes per 8 mm section of tissue. No caspase 3–positive suprabasal keratinocytes or dermal fibroblasts were observed following antibiotic treatments or in the cycloheximide-treated positive control (data not shown). Taken together, these results show

that treatment with the antibiotic mafenide induces loss of glycogen stores in tissue-engineered human skin substitutes but does not appear to trigger apoptosis through a caspase 3–dependent pathway.

DISCUSSION

Tissue-engineered skin was the first bioengineered organ to be used clinically.²⁰ Cell sourcing and the response of living human skin substitutes to adjuvant therapies remain significant challenges for the field of tissue engineering.² In this study, clinically used therapeutic concentrations of antimicrobial solutions were applied topically and dermally on fully stratified human skin substitutes. Our approach was to simulate clinical antibiotic application on meshed split-thickness skin grafts in which the epidermal surface of the graft receives the topical application and the interstices of the mesh graft permit dermal penetration of the antibiotic solutions. We show that clinically relevant dosages of mafenide applied to the epidermal or dermal surfaces of skin substitutes result in loss of viability accompanied by phenotypic changes indicative of cellular stress and loss of tissue

TABLE 1. ACTIVE CASPASE 3–POSITIVE CELLS FOLLOWING 48 h DERMAL OR TOPICAL TREATMENT WITH ANTIBIOTICS

	<i>NIKS active caspase 3–positive cells/8 mm</i>		<i>Primary human keratinocytes active caspase 3–positive cells/8 mm</i>	
	<i>48 h dermal application</i>	<i>48 h topical application</i>	<i>48 h dermal application</i>	<i>48 h topical application</i>
No antibiotic	1.5 (1, 2)	2.5 (4, 1)	4 (2, 6)	1.5 (3, 0)
Neo/poly	3.5 (4, 3)	5.5 (5, 6)	6 (7, 5)	1 (1, 1)
Mafenide	0 (0, 0)	7 (7, 7)	11 (12, 10)	2.5 (4, 1)

Values represent the average number of cells that stain positively for active caspase 3 per 8 mm tissue section. Actual values per tissue section are presented in parenthesis. Statistical significance determined using one-way ANOVA with Bonferroni's multiple comparison posttest ($p < 0.05$ for each treatment vs. control).

integrity, whereas neo/poly had little effect on viability and tissue morphology. These findings suggest that neo/poly is the antibiotic solution of choice for maintaining the viability and functionality of human skin substitutes.

The dramatic phenotypes exhibited by mafenide-treated epidermal keratinocytes in human skin substitutes have not been previously described. Earlier studies reported only that mafenide inhibited monolayer growth of several cell types, including keratinocytes.^{21–24} The mafenide-induced perinuclear vacuolization and ballooning degeneration observed in our studies are also histological features of skin keratinocytes associated with certain nutritional deficiencies, and a wide variety of acute stress such as viral infections.^{17,25} The differences seen in cell viability and morphology with dermal versus topical application may be related to the effective concentration of mafenide reaching the metabolically active keratinocytes either through the dermal component or the stratum corneum. It is important to consider both modes of application in light of the clinical use of mafenide on meshed split-thickness skin grafts where the antibiotic solutions come into direct contact with the dermal component and the cornified layer of the tissue.

A link between stress and glycogen storage has long been established in skin biology.¹⁶ Keratinocytes in intact human skin contain little to no glycogen, although glycogen is present in neonatal skin and upon injury in adult keratinocytes.^{16,26} The accumulation of glycogen following skin injury is hypothesized to result from a slowing in metabolic processes.²⁶ However, this link between keratinocyte glycogen content and mafenide-induced cytotoxicity was not observed in our studies. In contrast, we found that glycogen stores were depleted following exposure to mafenide but not neo/poly. The mechanism of mafenide-induced effects on glycolysis and gluconeogenesis in human keratinocytes is not known. Mafenide contains the sulfonamide functional group present in all sulfonamides; however, Eagon and McManus showed that mafenide's mechanism of action is different from that of other sulfonamides.²⁷ Cutaneous drug reactions to antibiotic sulfonamides are well documented and involve immune cells and mediators.²⁸ The mechanisms responsible for adverse reactions to sulfonamides have not been fully resolved.²⁹ Since our skin substitutes do not contain immune cells, mafenide most likely induces keratinocyte cytotoxicity in a cell autonomous manner. Oxidative metabolism of sulfonamides in keratinocytes has been shown to create cytotoxic-reactive hydroxylamine metabolites and drug-protein adducts.³⁰ It is possible that the stress response observed in our skin substitutes is the cytotoxic effect of the reactive species and haptenated cellular proteins.

A novel finding revealed by these studies is the loss of keratinocyte cell-substratum attachment following mafenide treatment of tissue-engineered human skin. In both organotypic and monolayer culture, mafenide-induced effects on adhesion were specific to cell-substratum adhesion. The decrease in cell viability with mafenide treatment cor-

relates with the extent of basal keratinocyte detachment from the substratum with both NIKS and primary human keratinocytes. The loss of basal keratinocyte adhesion to the underlying dermis is an important new finding that may explain mafenide-induced decreases in engraftment of skin substitutes on athymic mice.³¹ Anoikis, adhesion-mediated apoptosis, is triggered in numerous epithelial cell types by loss of cell-cell and cell-substratum contacts.^{32–35} We speculated that mafenide treatment may initiate a caspase-dependent apoptotic cascade by triggering the loss of cell-substratum adhesion in our tissue-engineered human skin substitutes. However, the low numbers of keratinocytes possessing activated caspase 3 lead us to conclude that caspase 3-dependent apoptosis is not triggered by mafenide-induced loss of cell-substratum contact. This finding is consistent with recent studies showing epidermal keratinocytes, unlike other epithelial cell types, are resistant to apoptosis following detachment from the basement membrane.³⁶ Maintenance of E-cadherin-mediated cell-cell contacts and the AKT pathway are considered to be key for survival of differentiating keratinocyte during the stratification process. Consistent with these studies, we found that mafenide-treated human skin substitutes and monolayer keratinocyte cultures exhibited loss of cell substratum attachment although cell-cell attachment remained intact. A more thorough analysis of mafenide's effect on keratinocyte differentiation and cell death is necessary to understand the pathways affected by this potent antibiotic. A number of questions remain unanswered regarding the impact of clinically used antimicrobials on the ability of tissue-engineered human skin substitutes to recover and maintain biological function following exposure to therapeutic doses of antibiotics such as mafenide.

In summary, mafenide and neo/poly remain valuable for the control of wound infection, particularly in patients with third-degree burns.³⁷ The results presented here are the first to identify the morphological and metabolic changes in human skin substitutes following treatment with therapeutic levels of mafenide. Unlike mafenide, topical and dermal application of the antibiotic combination neo/poly was nontoxic and had no effect on tissue morphology. The antibiotic-induced epidermal changes observed in our studies may compromise the biological function and ultimately impact the delivery of growth factors by clinically applied human skin substitutes. Our studies highlight the importance of prospectively identifying and understanding the effects of combination therapies comprised of complex tissue-engineered biological therapies and pharmaceutically active agents such as antimicrobials. There is a need to identify and evaluate new antimicrobial agents in response to the emergence of resistant microorganisms. In addition to the therapeutic use of tissue-engineered human skin substitutes in the treatment of severe skin loss, they may also provide an important screening platform to assess the cytotoxicity of emerging topical antimicrobials prior to clinical use.

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