

Streptolysin O enhances keratinocyte migration and proliferation and promotes skin organ culture wound healing in vitro

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ABSTRACT

ML-05, a modified form of the hemolytic and cytotoxic bacterial toxin, streptolysin O, is currently being investigated as a treatment for collagen-related disorders such as scleroderma and fibrosis. Furthermore, ML-05 may be effective in promoting wound healing and alleviating the formation of hypertrophic scars and keloids. To investigate the effects of ML-05 on wound-healing processes, in vitro wound-healing scratch assays (using human primary epidermal keratinocytes and dermal fibroblasts) and a human skin organ culture wound model were utilized. ML-05 markedly enhanced keratinocyte migration and proliferation in wound scratch assays. ML-05 did not affect either proliferation or migration of dermal fibroblasts, indicating that ML-05's effects on cell migration/proliferation may be keratinocyte-specific. ML-05 was tested in a dose-dependent manner in a skin organ culture wound model using two different application methods: Through the culture media (dermal exposure) or direct topical treatment of the wound surface. ML-05 was found to accelerate wound healing as measured by reepithelialization, particularly after topical application. Therefore, ML-05 may have potential as a wound-healing agent that promotes reepithelialization through stimulation of keratinocyte migration and proliferation.

Streptolysin O (SLO) is a hemolytic exotoxin produced by *Streptococcus* species of groups A, C, and G. This single-chain protein (60–70 kDa) can, as a thiol-activated, cholesterol-binding agent, form pores in cell membranes in its reduced state but does not readily form them postoxidation. As red blood cells are particularly susceptible to cytolysis, they are used to determine the cytolytic properties of SLO. Structurally, the SLO primary gene product is made up of 571 amino acids with several structural and functional domains including a cholesterol-binding domain. Similar toxins are produced by other species of Gram-positive bacteria.^{1–4} ML-05 is a nonhemolytic, non-cytotoxic form of SLO produced by oxidation of the parent molecule. It is undergoing preclinical development as a possible treatment for diseases and conditions involving abnormal or excessive collagen deposition.^{5,6} The rationale for testing ML-05 as a stimulator of wound healing initiates from earlier studies of treatment for symptoms of motor deficit disorders⁷ during which some patients who were being treated with ML-05 observed reductions in the appearance of scarring and wrinkles as well as enhanced healing after surgery⁵ (J. McMichael, personal communication). Subsequently, it was reported that ML-05 could modulate or reduce the excessive production of collagen in two murine models of scleroderma: the genetically based tight skin model and the bleomycin-induced scleroderma model.⁶ Fibrogenesis (the formation of hypertrophic scars (HSs) and keloids) during wound healing is related to

abnormal collagen deposition.^{8–11} Accordingly, it was hypothesized that ML-05 may be useful in modulating or reducing collagen deposition during the wound healing process. ML-05 may indirectly or directly alter collagen production to facilitate the restoration of normal extracellular matrices (ECMs) within tissues during wound healing via several possible mechanisms. First, ML-05 may have beneficial immunomodulatory properties related to both collagen production and wound healing. SLO and related substances can induce the production of a variety of cytokines and chemokines.^{12–16} As an example of the favorable effects of cytokines on wound-healing processes, interferon- γ and interleukin-12 (IL-12) have been shown to decrease abnormal collagen production associated with fibrogenesis in wound healing.^{10,17–20} Second, SLO and related compounds may increase or maintain the expression of cell surface receptors that are involved in ECM organization, notably hyaluronan receptor CD44.^{6,21–23} Appropriate changes in CD44 levels and subsequent downstream events within the ECM may promote wound healing.^{24–26} Finally, at sublytic concentrations, pneumolysin, a bacterial protein chemically related to SLO, induced collagenase production in fibroblasts.²⁷ Increased collagenase levels may have local effects on abnormal collagen production and deposition.²⁸

The pharmacological effects of low levels of ML-05 on keratinocyte growth and up-regulation of CD44 were characterized in previous in vitro studies.⁶ In this report,

the effects of ML-05 on keratinocytes and epithelialization were investigated in two different wound-healing assays. ML-05 was evaluated for its effects on in vitro migration and proliferation of normal human epidermal keratinocytes and primary human dermal fibroblasts (HDFs) in wound scratch assays, and for its ability to promote the healing of human skin explant cultures wounded by punch biopsy.

MATERIALS AND METHODS

SLO and ML-05

SLO for these studies was purchased from Sigma Chemicals, St. Louis, MO (catalog number S5265; 25,000–50,000 U/vial). Oxidized SLO was prepared by bubbling air or oxygen into solutions of SLO for defined time periods. Evidence of oxidation was obtained from the lack of activity of a given preparation in a standard hemolysis assay provided by Sigma Chemicals, except that sheep red blood cells were used instead of human red blood cells. Stock solutions of the final formulation (ML-05) for in vitro experiments, containing 2,000 U/mL (unit value based on hemolytic activity of starting material), were prepared in phenolated saline or deionized water, filtered through a 0.22 μm filter, and stored at 4 or -20°C . Furthermore, these preparations were tested for cytotoxicity or growth inhibition against a variety of cell lines (normal human epidermal keratinocytes, normal human diploid fibroblasts, and certain established cell lines). No cytotoxicity was observed at final test concentrations as high as 50 U/mL.⁶

Keratinocyte and fibroblast migration/proliferation assay

Primary human keratinocytes were grown to 80% confluency as previously described.^{29,30} Primary HDFs were provided by M. Simon (SUNY, Stony Brook, NY). Cells were grown in Dulbecco's-modified Eagle's medium (DMEM) containing 10% fetal bovine serum. At 24 hours before the experiment, keratinocytes were transferred to basal KBM medium (GIBCO-BRL, Grand Island, NY), whereas fibroblasts were transferred to the DMEM with 5% stripped serum as described.³¹ Before the scratch, one set of culture dishes was treated with 10 $\mu\text{g}/\text{mL}$ Mitomycin C (ICN Biomedicals, Emeryville, CA) for 2 hours (to inhibit cell proliferation and measure migration only) and washed with basal media. A second set of culture dishes, maintained in the absence of mitomycin C, was used to evaluate both keratinocyte proliferation and migration. Scratches were performed as previously described.^{31,32} Cells were incubated with four different concentrations of ML-05 (0.02, 0.2, 2, and 20 U/mL). Phosphate-buffered saline solution (PBS) was used as a negative control (untreated cells). The same fields were rephotographed 24 and 48 hours later, and cell migration was quantified as previously described.^{31–33} Fifteen measurements were taken for each experimental condition, and distance coverage by cells moving into the scratch wound area was quantified. Three images were analyzed per condition, per time point, and averages and standard deviations were calculated. Statis-

tical analyses (2-tailed *T* test) were performed and significance was established from calculating *p*-values.

Human skin organ culture wound assay and histology

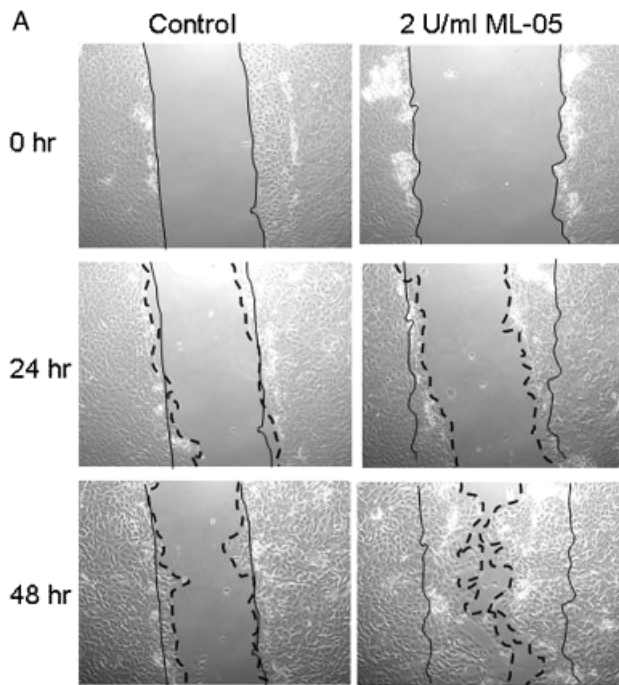
Wound assays were conducted using previously described methods.³² Specimens of normal human skin obtained from reduction mammoplasty (Institutional Research Board-approved protocol H#9796-03) were maintained at the air-liquid interface using keratinocyte basal medium (GIBCO-BRL). Specimens were divided into two groups: control and wounded. Skin samples, 1 cm^2 each, were wounded by the creation of 4 mm punch biopsies in the center through the reticular dermis. Skin samples were incubated with four different concentrations of ML-05 using two different treatment conditions. Indirect treatment of the epidermis through the dermis (dermal exposure) was achieved by adding ML-05 to the medium (final concentrations, 0.02, 0.2, 2, and 20 U/mL of medium). Direct treatment of the wound surface (topical exposure) involved the topical application of ML-05 to the wound site (solutions of 0.02, 0.2, 2, and 20 U/mL). A small amount of ointment was placed at the border of the skin sample (not the wound) to prevent leakage to the culture medium below. PBS was used as a negative control for both types of treatments. For each condition, two experiments were conducted: one with explants being evaluated over 4 days posttreatment, and a second with explants evaluated over a 6-day period. Wounds were quantified daily by planimetry as described.³² The edge of each wound was traced, and lab members blinded to the experiment calculated the surface area for each wound for each time point and treatment condition. Each experiment was conducted in quadruplicate for all testing conditions using skin specimens obtained from the same donor.

All specimens were collected and frozen sections were prepared using OCT compound (Tissue Tek, Sakura Finetek, U.S.A., Inc., Torrance, CA). Five-micrometer thick sections were stained with hematoxylin & eosin. The sections were analyzed using a Nikon microscope and digital images were obtained using a Spot RT camera (Diagnostic Instruments, Sterling Heights, MI) as previously described.^{31–32}

RESULTS

ML-05 stimulates keratinocyte migration and proliferation

Two major cellular processes mark the biology of the keratinocytes that participate in wound healing: migration and proliferation. To test whether ML-05 affects keratinocyte migration, a wound scratch assay was utilized. Primary human keratinocytes (pretreated with mitomycin C to inhibit proliferation) were wounded by a scratch, and incubated in the presence or absence of 0.02, 0.2, 2, and 20 U/mL of ML-05. Cell migration was monitored and quantified 24 and 48 hours after the scratch. It was found that after 24 hours, and more significantly, after 48 hours, ML-05 stimulated keratinocyte migration (Figure 1). At 48 hours postscratch, migration in cells treated with 0.02–2 U/mL increased in a concentration-dependent manner



B

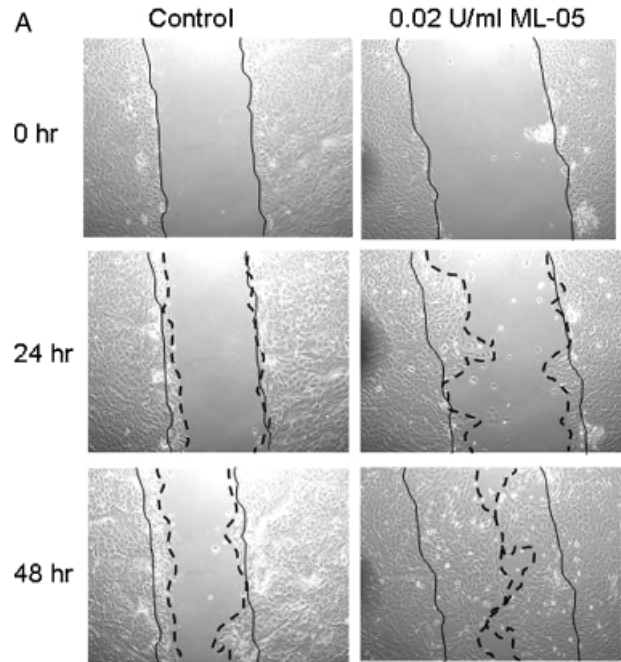
% Wound width coverage		
ML-05 U/ml	24 hrs	48 hrs
0	5.0 ± 1	16.0 ± 2
0.02	8.7 ± 1 *	32.6 ± 1 *
0.2	6.1 ± 3	58.0 ± 4 *
2	36.7 ± 3 *	81.6 ± 2 *
20	21.3 ± 2 *	40.4 ± 1 *

+ mitomycin C

Figure 1. ML-05 promotes keratinocyte migration. (A) Results of a typical scratch assay are shown. Keratinocytes were pre-treated with Mitomycin C. Initial scratch is shown with a full line, whereas the migrating front is marked by a broken line; 0.2 U/mL of ML-05 stimulated migration. (B) Table represents quantitative analyses of cellular migration for all four ML-05 concentrations. Values are averages of 15-independent measurements of experiments performed in triplicate. The gray area highlights the concentration that was the most effective in promoting migration. *Statistically significant changes $p < 0.001$.

from 33 to 82%, as opposed to an increase of 16% in untreated control cultures. Migration at the highest (20 U/mL) test concentration increased only to 40%.

To test whether ML-05 affected keratinocyte proliferation, similar experiments were performed with cells that were not treated with Mitomycin C, thus allowing cellular proliferation in addition to migration. It was found that ML-05 promoted keratinocyte proliferation as well as migration (Figure 2). At 48 hours postscratch, proliferation within the scratch zones in untreated control cultures increased 40%, whereas in cultures treated with ML-05 at test



B

% Wound width coverage		
ML-05 U/ml	24 hrs	48 hrs
0	15.0 ± 1	40.0 ± 2
0.02	23.9 ± 2 *	87.0 ± 3 *
0.2	29.0 ± 2 *	75.0 ± 4 *
2	8.3 ± 1 *	38.9 ± 3
20	12.2 ± 1	20.4 ± 1 *

- mitomycin C

Figure 2. ML-05 promotes keratinocyte proliferation. (A) The effects on keratinocytes untreated with Mitomycin C are shown. (B) Table represents quantitative analyses of cellular migration for all four ML-05 concentrations. Values are averages of 15-independent measurements of experiments performed in triplicate. Gray area indicates the concentration that was the most effective in promoting proliferation. *Statistically significant changes $p < 0.001$.

concentrations of 0.02 and 0.2 U/mL, coverage of scratches was increased by 87 and 75%, respectively. This effect disappeared with increasing ML-05 concentration. In fact, with 20 U/mL of ML-05, proliferation at 48 hours was reduced relative to that of the untreated control culture.

ML-05 does not affect migration and/or proliferation of dermal fibroblasts

To test whether the ML-05 effect on cellular migration/proliferation is either a general phenomenon or keratinocyte-specific, wound scratch assays were performed on

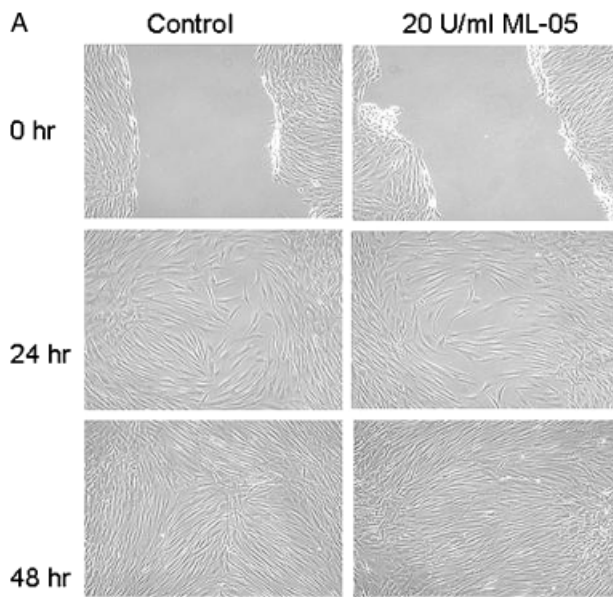


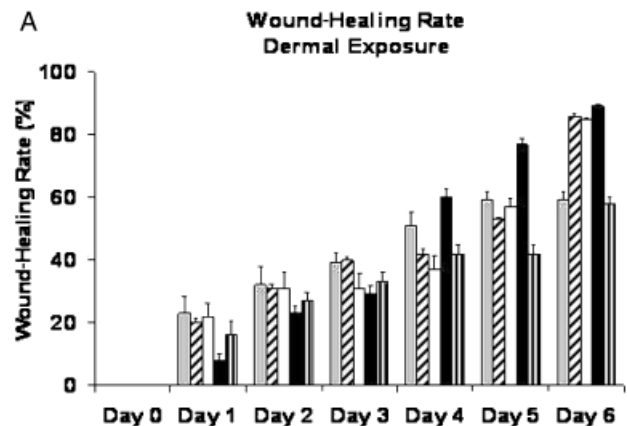
Figure 3. ML-05 does not affect fibroblast migration. Primary human dermal fibroblasts were pretreated with Mitomycin C. There is no significant effect on ML-05 treated cells compared with untreated control.

primary HDFs. It was found that ML-05 had no effect on either fibroblast migration (using cells pretreated with Mitomycin C) or proliferation/migration (no Mitomycin C pretreatment). No noteworthy differences were found in scratch coverage between primary HDFs treated with ML-05 at all test concentrations and untreated control cells (Figure 3).

ML-05 promotes wound healing in human skin organ cultures

Based on the results presented above, it was hypothesized that ML-05 promotes wound healing by promoting wound reepithelialization. To test this hypothesis, a wound-healing organ culture model was used. This model allowed for following reepithelialization from the epidermal perspective, i.e., in the absence of circulating factors and inflammatory reactions. Normal skin was wounded by a 4 mm biopsy punch and maintained at the air-liquid interface in the presence or absence of ML-05. Dual delivery methods were tested: indirect (through the dermis, by adding ML-05 to the medium) or direct (by topical application to the wound site). Wound healing was monitored on a daily basis for a period of 4–6 days, and wound healing was quantified on days 4 and 6 by planimetry. In addition, duplicate experiments were frozen, sectioned, and stained for evaluation of the healing process.

It was found, both by quantification of wound healing (Figure 4) and by histological evaluations (Figure 5), that wounds on the 4th and 6th day of indirect (dermal exposure) ML-05 treatment reepithelialized modestly faster than untreated controls. Reepithelialization in untreated control cultures ranged from 43 to 47% and from 56 to 62%, in the 4- and 6-day experiments respectively, whereas



B

ML-05 U/ml	4 days	6 days
0	45± 2	59± 3
0.02	69± 1	86± 1
0.2	37± 5	85± 1
2	58 ± 2	89± 1
20	41± 4	58± 2

Figure 4. ML-05 stimulates reepithelialization when applied through dermis. (A) Quantitative analyses of rates of wound healing taken each day during the period of 6 days. (B) Table shows summary of the healing rates at days 4 and 6 for all four ML-05 concentrations. Values are averages of results of tests performed in quadruplicate.

reepithelialization in the presence of 0.02–20 U/mL of ML-05 ranged from 37 to 69% and from 58 to 89% in the 4- and 6-day experiments, respectively (Figures 4 and 5). Histological examinations of the day 6 dermal exposure cultures treated with ML-05 showed essentially complete closure and a fully restored epidermis compared with the incompletely healed untreated control (Figure 5).

In contrast to dermal exposure, topical treatment yielded markedly enhanced reepithelialization of treated wounds relative to untreated controls in both the 4- and 6-day experiments (Figures 6 and 7). Reepithelialization ranged from 29 to 35% and from 31 to 37% in untreated control cultures in the 4- and 6-day experiments, respectively. In the presence of 0.02–20 U/mL of ML-05, epidermal regrowth ranged from 52 to 67% and from 60 to 100% in the 4- and 6-day experiments, respectively. Activity of ML-05 was optimal at the two lowest test concentrations, 0.02 and 0.2 U/mL, which was similar to the results obtained from scratch assays. There was 100% closure in the days 5 and 6 samples treated with ML-05 at 0.2 U/mL. Microscopic skin sections evaluated histologically (Figure 7) showed incomplete wound healing in controls, whereas topical application of the two lowest test concentrations of ML-05 (0.02 and 0.2 U/mL) showed a fully restored epidermis. However, at the two higher concentrations (2 and 20 U/mL), incomplete healing was

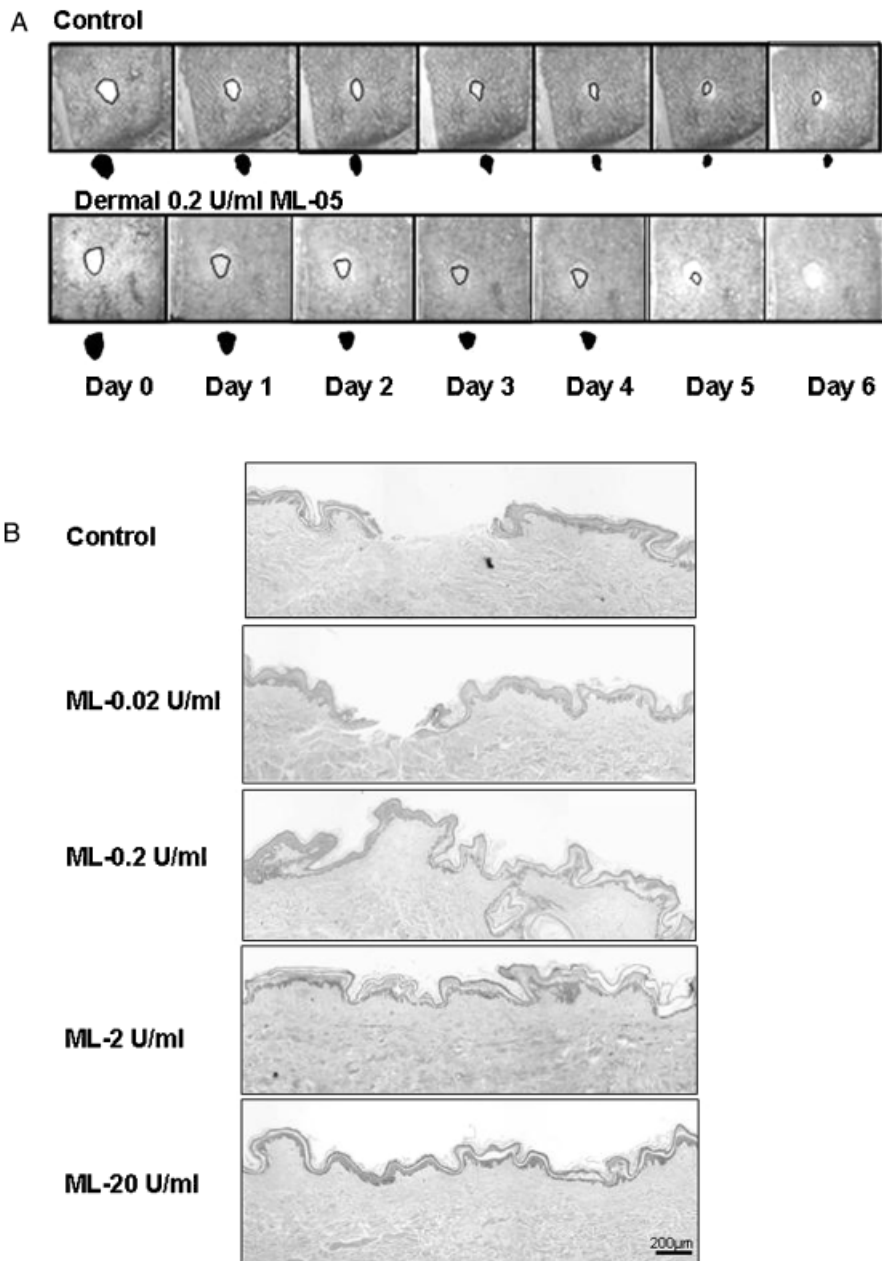


Figure 5. ML-05 stimulates reepithelialization when applied through dermis. (A) Macroscopic view of typical organ culture wounding experiment. Circles below each photograph represent the trace of the actual wound. (B) Typical histology is shown demonstrating complete reepithelialization at 0.2 U/mL of ML-05.

observed, with a fragile epithelium, showing loss of attachment of suprabasal keratinocytes to the basal layer.

DISCUSSION

The results presented in this paper indicate that ML-05 is capable of promoting epithelialization as measured by proliferation and migration in keratinocyte scratch assays, as well as promoting healing in a skin organ culture wound model. Keratinocyte migration and proliferation are important steps in the wound-healing process.^{34–36} Although ML-05 increased both keratinocyte proliferation and migration, the effects are distinguished at different doses of

ML-05. The effect on migration was most prominent at 2 U/mL, whereas proliferation was most affected at 0.02 U/mL. This suggests that improved proliferation and migration were distinct concentration-dependent effects of ML-05 treatment. This was not surprising, as it was noted experimentally (Lee B., Stojadinovic O., Vouthounis C., Tomic-Canic M. Transcriptional profile of acute wound healing process, manuscript in preparation) as well as in the literature that the mechanisms of keratinocyte migration and proliferation are independent of each other and that they participate independently of each other during the wound-healing process.^{35,37} With respect to concentration-dependent effects, it was observed that both migration and

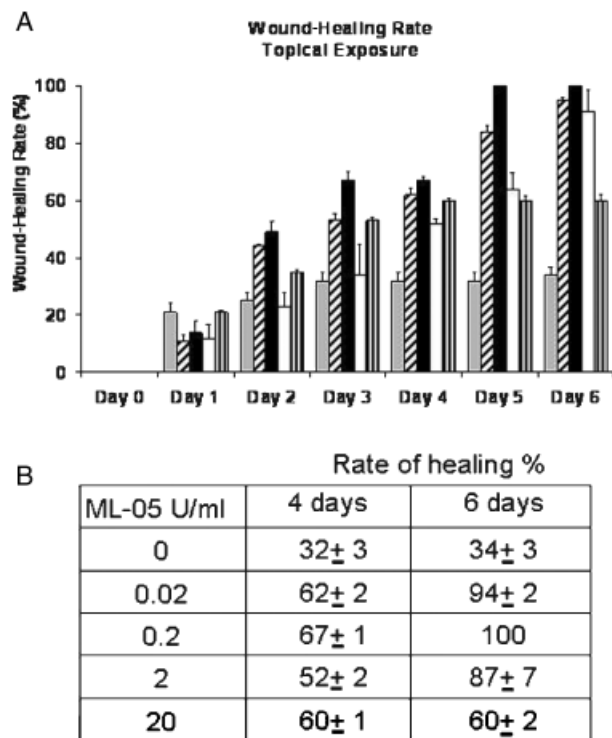


Figure 6. Topical ML-05 stimulates reepithelialization. (A) Quantitative analyses of rates of wound healing taken each day during the period of 6 days. (B) Table shows summary of the healing rates at days 4 and 6 for all four ML-05 concentrations. Values are averages of results of tests performed in quadruplicate.

proliferation were better at lower concentrations (0.02–0.2 U/mL) than at higher concentrations (2–20 U/mL). In fact, proliferation at 20 U/mL was inhibited relative to that of the corresponding negative controls. Growth inhibition or cytotoxicity assays were not conducted in the current studies, but ML-05 at concentrations of 0.02–2 U/mL had no effects on the growth of primary keratinocytes for up to 10 days in a previous study.⁶ Moreover, in conventional cytotoxicity assays involving several normal and transformed cell lines, ML-05 had no cytotoxic effects at concentrations as high as 50 U/mL (Mamber and McMichael, unpublished data). The nonlinear (bell-shaped) dose–response relationships for ML-05 with respect to keratinocyte migration and proliferation are suggestive of the phenomenon known as chemical hormesis, in which low doses of a compound stimulate, and high doses inhibit, beneficial biological activities of interest.³⁸ In addition, no cell death or related pathogenic changes were observed on histology of human skin treated with ML-05 for 6 days (see Figs. 5 and 7).

In order to determine the cell specificity of the effects of ML-05 on migration and proliferation, the effects of ML-05 on primary human keratinocytes and dermal fibroblasts were compared. It was found that the effects of ML-05 were keratinocyte-specific, i.e., no enhanced migration or proliferation effects were detected on fibroblasts. This was surprising because fibroblasts are responsible for produ-

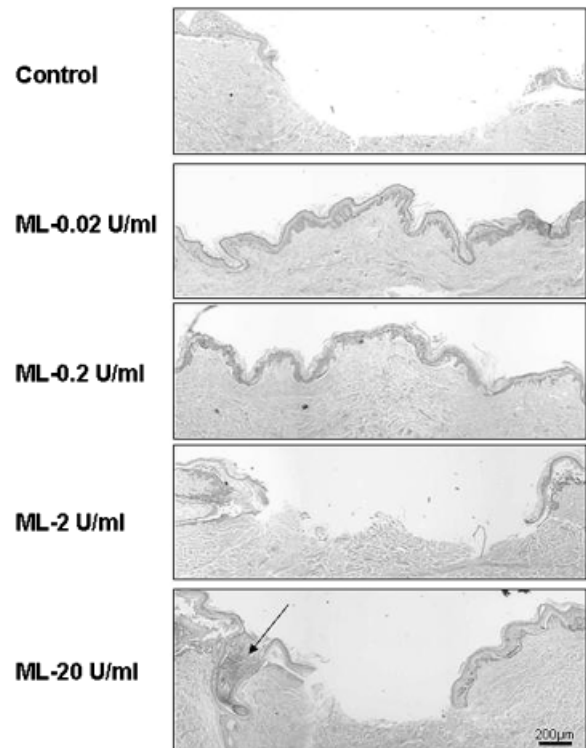


Figure 7. Topical ML-05 stimulates reepithelialization. Histology of wounds treated topically with PBS (control) or ML-05 is shown. Reepithelialization is completed at concentrations 0.02 and 0.2 (middle panel). Interestingly, reepithelialization was incomplete at higher concentrations (lower panels) and cornified cysts were found (arrow).

cing excessive collagen during the formation of HSs as well as in fibrotic diseases.^{8,39,40} Johnson et al.²⁷ reported that pneumolysin induced collagenase production in fibroblasts, and Arakawa et al.²⁸ reported that collagenase production by fibroblasts may have a role in lessening excessive accumulation of collagen related to HS formation. Accordingly, it was expected that ML-05 could directly affect fibroblasts to modulate excessive collagen production during wound healing. However, the lack of increased fibroblast migration/proliferation by ML-05 in these studies, coupled with experiments indicating that collagenase production is not induced in normal human diploid fibroblasts treated with ML-05 *in vitro* (unpublished observations), suggests that ML-05 does not directly modulate collagen production by fibroblasts. *In vivo* investigations of ML-05 activity in scleroderma and fibrosis used modulation or reduction of collagen production as a biological endpoint.⁶ Given the *in vitro* results reported thus far in experiments with fibroblasts, it appears more likely that any effects of ML-05 on collagen production itself are indirect. Indeed, while ML-05 did not affect the rates of fibroblast migration and/or proliferation, the spatial orientation of fibroblasts within the scratch zones that were treated with ML-05 appeared to be more orderly than that of the comparable control cell cultures. Although this observation requires further study, it indicates that ML-05 may contribute to improved ECM organization during the

wound-healing process, which may potentially lessen the formation of HSs and keloids.

Despite the lack of increased fibroblast migration, ML-05 may affect migration of other types of cells that may be involved in wound-healing responses, such as neutrophils (polymorphonuclear leukocytes). Interestingly, both SLO and pneumolysin have been reported to inhibit neutrophil migration.^{41,42} In contrast, Johnson et al.⁴³ reported that low concentrations of pneumolysin stimulated neutrophil migration. Neutrophils and keratinocytes have been shown to interact in wound-healing processes.^{44,45} Consequently, ML-05 may, by enhancing neutrophil migration in addition to keratinocyte proliferation and migration, represent a substance that has multiple beneficial functions in promoting wound healing and reducing HS formation. Furthermore, several investigators have indicated that neutrophils and keratinocytes can cooperate in mediating resistance to microbial infections.^{46,47} This raises the intriguing possibility that stimulation of both keratinocyte and neutrophil migration by ML-05 can promote resistance to, and perhaps aid healing of, these infections.

The results of ML-05 effects in skin organ culture wound model are well correlated with those obtained in scratch assays. It is rather interesting that the most prominent effects were found with a low concentration (0.02–0.2 U/mL) after topical treatment, whereas concentrations greater than 0.2 U/mL showed incomplete healing with areas of encapsulated cornification (Figure 7 arrows). There are two possibilities for the observed effect. Keratinocytes treated with the higher topical concentrations of ML-05 may have migrated too fast, resulting in the improper formation of cellular junctions. Alternatively, high concentrations of topical ML-05 may either have inhibited cell adhesion or induced premature cornification. As with the migration and proliferation assays, the low-dose stimulatory effects and high-dose incomplete healing effects of ML-05 with respect to the skin organ wound-healing model are suggestive of chemical hormesis. In future *in vitro* wound-healing experiments, it will be necessary to test ML-05 concentrations in a narrower range than 0.02–20 U/mL in both experimental models in order to determine the optimal range of effective test concentrations.

Cellular mechanisms by which ML-05 can facilitate wound healing also require further investigation. As a potential immunomodulator, ML-05 could be affecting cytokine/chemokine expression, neutrophil and epithelial cell migration/proliferation, and other specific responses relevant to wound healing.^{10,13,14,19,20} A variety of cytokines and chemokines are known to have a role in promoting keratinocyte migration, proliferation, and reepithelialization during wound healing.^{37,48–51} The production of several of these cytokines and chemokines can be induced by SLO and related substances.^{12–16}

In addition to, or perhaps in conjunction with, its potential immunomodulatory properties, ML-05 may benefit the wound-healing process by inducing or maintaining the expression of hyaluronan receptor CD44 in keratinocytes. ML-05 was previously shown to activate CD44 expression in keratinocytes *in vitro*.⁶ Up-regulation of keratinocyte CD44 could lead to the accumulation of CD44 in the ECM via proteolytic cleavage, which can then promote the assembly of hyaluronan-rich ECMs through the formation of CD44–hyaluronan acid complexes.^{23,52} These complexes

could modulate epithelial cell behavior, including increased mobility, altered adhesion, and/or altered proliferation of keratinocytes. Accordingly, such physiological changes occurring in the area of the wound may ultimately result in a restoration of normal ECM organization in that area.^{23–26,52}

In conclusion, low concentrations of ML-05 promoted healing and reepithelialization in human skin cultures wounded *in vitro* by punch biopsy. ML-05 also promoted proliferation and migration of normal human epidermal keratinocytes but did not improve that of normal HDFs. The results of the present research suggest that in addition to previously shown activity in alleviating diseases involving excess collagen deposition, such as scleroderma,⁶ ML-05 may promote wound healing.

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