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Laminin 511-E8 Fragment Improves Second-Degree Burn Wound Healing in a Rat Model

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Abstract

Background: The rate of re-epithelialization is the primary determinant of the morbidity and mortality in patients with severe burn injuries. Laminin $\alpha 5\beta 1\gamma 1$ (LM511) is an extracellular structural protein that can support epithelial cell adhesion and migration. LM511-E8 is a functionally minimal form of LM511 with an efficacy similar to that of the full-length protein. To investigate whether treatment of burn wounds with the LM511-E8 fragment improves wound closure in a rat second-degree burn wound model.

Methods: Second-degree burn wounds were produced in vivo on the backs of rats. The rats were separated into saline-treated control and LM511-E8-treated groups (n=9 per group),

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which were imaged on day 0 and on days 7, 14, 21 and 28 post-injury. Tissue sections were processed for histological and immunochemical examination and scored based on the overall pathology, epithelialization and presence of cytokeratin 10 and 14.

Results: Burn wound healing improved in the LM511-E8-treated group compared with that in the control group from 7-28 days post-wounding ($P < 0.01$). The re-epithelialization of the LM511-E8-treated group was significantly faster than that of the control group at 7-28 days post-injury, with the largest improvement observed on days 7 and 14 ($P < 0.001$). The overall pathological score of the LM511-E8-treated group was higher than that of the control group at 14-28 days post-injury; maximum improvement was observed on days 14 and 21.

Conclusion: The use of LM511-E8 is a promising therapeutic option for managing second-degree burns.

Keywords

Burns; Epithelialization; Laminin 511-E8; Wound Healing

Introduction

The rate at which burns heal following injury is a primary determinant of the likelihood of infection and slow healing is associated with prolonged hospital stays and severe patient morbidity. Development of new methods to improve the rate of wound repair and thereby improve outcomes would substantially benefit patients. One approach is the direct application of biologically derived proteins, peptides or compounds to the wound bed, where the applied material is known to be involved in the native wound healing response. Keratinocytes, the primary cells of the epidermis, are important not only for their role as physical barriers to the body, but also for rebuilding the injured layer via re-epithelialization [1]. This process involves keratinocyte proliferation and interaction with a provisional extracellular substrate to migrate to the wound bed, thereby closing the wound [2]. In this study, we hypothesized that an extracellular matrix component that is known to promote keratinocyte adhesion and migration could aid the re-epithelialization process, thereby improving the rate of wound healing.

Laminins (LMs) are extracellular macromolecules that play key roles in the regulation of a plethora of core cell and tissue functions, including regulation of cell proliferation, adhesion and migration [3]. Indeed, LMs are involved in most tissue remodeling processes, including neovascularization processes during wound repair [4]. Each LM is an obligate heterotrimer comprised of an α , β and γ subunit with each subunit derived from a separate gene. Eighteen different $\alpha\beta\gamma$ isoforms of LMs have been identified [4,5]. Different LM isoforms display distinct distribution profiles and differ in terms of their structure and in their specificity and

affinity for cell surface receptors. For example, laminin $\alpha 5\beta 1\gamma 1$ (LM511) potently enhances keratinocyte migration in *in-vitro* wound healing assays and *in-vitro* and *in-vivo* assays have demonstrated that LM511 provides a robust culture platform for the expansion of epidermal keratinocytes [6]. LM511 is also located in the endothelial basement membrane and supports faster migration of cultured endothelial cells [7-9]. As each of these activities are associated with the native wound response, supplying recombinant LM511 at wound sites may promote re-epithelialization and angiogenesis [10,11].

The LM511 E8 protein fragment (LM511-E8) is derived via elastin-mediated proteolytic processing of the C-terminus of LM511. The fragment comprises of the C-terminal regions of the α , β and γ chains, which include the high-affinity integrin-binding sites within the laminin globular domains (Fig. 1) [12,13]. Thus, although LM511-E8 is substantially smaller (~150 kDa) than the full-length protein (~800 kDa), it serves as a functionally minimal form of LM511 and retains the key characteristics of the full-length protein including integrin-binding and dystroglycan binding sites that support adhesion and migration [9,14-16]. Recent studies demonstrated that in biosynthetic scaffold-based approaches for corneal epithelial tissue engineering, LM511-E8 supported human corneal epithelial keratinocyte regeneration with an efficacy similar to that of full-length LM511 and LM521 [17-19]. Therefore, using this smaller fragment may be sufficient for the effective treatment of burn wounds and have benefits over the full-sized protein in terms of future manufacturing scale-up and therapeutic delivery.

In this study, we investigated the feasibility of using LM511-E8 to repair tissues in burn wounds and its ability to promote wound closure and epithelialization at the wound edge.

Methods and Materials

Animal Experiments

Animal experiments were approved by the Animal Care and Use Committee of Chulalongkorn University (approval number: 003/2562 on 03/2019).

Eighteen eight-week-old male Wistar rats (150-180 g) were obtained from the Namura Animal Laboratory Center (Bangkok, Thailand). The rats were acclimatized for 7 days under a controlled 12-h light/dark cycle and were fed chow and provided ad libitum access to water. To create second-degree burns, rats were anesthetized using isoflurane and the dorsal skin was shaved. Two burn wounds were created on the back of each rat using a 10 mm diameter aluminum rod, which was heated to 100°C and applied to the skin for 10 s [20]. The rats were divided into two groups of 9 rats per group. Normal saline solution (100 μ L) or 2.5 μ g LM511-E8 (commercially available as iMatrix-511; Takara Bio Inc., Nippi, Tokyo, Japan) in 100 μ L Phosphate-Buffered Saline (PBS), was applied daily to each wound on the rats in the control and treatment groups, respectively.

For four animals/group (8 wounds), the wounds were imaged on days 0, 7, 14, 21 and 28 after injury and the wound areas were measured using the ImageJ software version 1.52t (NIH, Bethesda, MA, USA) using the freehand selection tool. Wound areas are reported as percentage area relative to day 0. For five animals/group, rats were euthanized after 3, 7, 14, 21 and 28 days, the wound tissues were excised, immediately fixed in 10% neutral-buffered formalin for at least 48 h then embedded in paraffin.

Histopathology and Immunohistochemistry

The formalin-fixed paraffin-embedded tissues were cut into 3 μ m sections using a Leica RM 2125 microtome (Leica, Heidelberg, Germany). Hematoxylin and eosin (H and E) staining was performed. In addition, immunohistochemistry of the tissue sections was performed using monoclonal antibodies against cytokeratin 10 (Dako Corp., Carpinteria, CA, USA) and cytokeratin 14 (Neomarkers, Inc., Fremont, CA, USA), followed by detection using a benchmark staining platform: ultraView Universal DAB for cytokeratin 10 and ultraView Universal Alkaline Phosphatase Red Detection Kit for cytokeratin 14 (Ventana Medical Systems Inc., Tucson, AZ, USA) [21].

Histological sections of the wounds were graded using a histopathological scoring system following the published method [22-26]. In addition, three dermatopathologists (authors MA, AS and JW), who were blinded to the treatment regime, independently scored the H and E - stained sections based on five criteria: epithelialization, Polymorphonuclear Leukocyte (PMNL) infiltration, collagen formation, the number of fibroblasts and presence of new blood vessels. A score of 0 was assigned where there was no evident epithelialization and no increase in the number of fibroblasts, PMNL or newly formed blood vessels. A score of 1 indicated an increased thickness of the edges of cut epithelial tissue sections or the presence of few fibroblasts, PMNL and small numbers of newly formed blood vessels; 2 indicated epithelial cell migration or the presence of moderate numbers of fibroblasts, PMNL and newly formed blood vessels; 3 indicated epithelial bridging of the incision or the presence of many fibroblasts, PMNL and newly formed blood vessels; 4 was assigned for sections where there was complete regeneration of the epithelium or when excessive numbers of fibroblasts, PMNL and newly formed blood vessels were present. The mean scores from the three scorers were determined and combined to give an overall pathology score. The anti-cytokeratin immunohistochemistry sections were scored in a similar manner, with three dermatopathologists scoring the sections on a scale of 0-4, starting from no staining to strong staining.

Statistical Analysis

Data were determined for normal distribution by the Shapiro-Wilk test ($P > 0.05$). For normally distributed data, one-way Analysis of Variance (ANOVA) followed by Bonferroni's post hoc

test were used to compare groups, or two-way mixed ANOVA was used to determine the difference between groups and times. For non-normally distributed data, the Kruskal-Wallis test was used to test the difference between groups. GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses. P-values < 0.05 were considered statistically significant.

Results

Treatment with LM511-E8 Increased Healing of Second-degree Burn Wounds

LM511-E8 has been extensively tested for its ability to support cell adhesion, migration and proliferation *in-vitro* [15-17]. We extended these studies by analyzing the effect of treatment upon Rat Dermal Fibroblast (RDF) scratch wound closure (Fig. 1). Mean migration velocities increased compared with controls when treated at concentrations of 1.0, 2.5 and 5.0 $\mu\text{g}/\mu\text{L}$, reaching statistical significance at 2.5 $\mu\text{g}/\mu\text{L}$ (Supplemental Figure 1b, mean $\pm$ SD for control, $0.76 \pm 0.07 \mu\text{m}/\text{min}$, LM511E8, 0.5 $\mu\text{g}/\mu\text{L}$ $0.75 \pm 0.23 \mu\text{m}/\text{min}$, 1.0 $\mu\text{g}/\mu\text{L}$ $0.89 \pm 0.17 \mu\text{m}/\text{min}$, 2.5 $\mu\text{g}/\mu\text{L}$ $0.99 \pm 0.082 \mu\text{m}/\text{min}$ and 5.0 $\mu\text{g}/\mu\text{L}$, $0.79 \pm 0.16 \mu\text{m}/\text{min}$; one-way ANOVA $F(4, 40) = 4.06$, $P = 0.007$ and post hoc $p=0.025$ for 2.5 $\mu\text{g}/\mu\text{L}$ for treated cells vs. control).

Next, rat back-skin second-degree burn wounds were treated with 2.5 μg LM511-E8 per wound per day or normal saline as control. Wound areas were measured at day 0 and days 7, 14, 21 and 28 after injury. Visually, the LM511-E8-treated wounds appeared to heal more rapidly than the saline-treated controls (Fig. 1). This observation was reinforced by measurements of the percentage wound closure, which were statistically significantly improved in the LM511-E8-treated wounds compared to the control at all time points (Figure 1c, % wound healing $\pm$ SD in control vs. LM511-E8-treated wound day 7: $15.6 \pm 0.9\%$ vs. $21.3 \pm 0.7\%$, $P < 0.001$; day 14: $57.8 \pm 0.4\%$ vs. $61.4 \pm 0.8\%$, $P < 0.001$; day 21: $74.7 \pm 1.1\%$ vs. $78.1 \pm 0.4\%$, $P < 0.01$; and day 28: $95.8 \pm 0.5\%$ vs. $99.1 \pm 0.5\%$, $P < 0.05$; two-way ANOVA).

One wound per time point at days 3, 7, 14, 21 and 28 was processed for histological and immunohistochemistry analyses (Fig. 2). Blinded scoring of the histology sections by the three dermatopathologists supported that the LM511-E8-treated animals had considerably improved wound response, with higher re-epithelialization than the saline-treated animals in the control group (Fig. 2, mean epithelialization score in control vs. LM511-E8-treated day 7: 1.7 vs. 3.0, day 14: 2.3 vs. 3.7, day 21: 3.0 vs. 4.0; day 28: 3.0 vs. 4.0). There were no differences in the histopathological scores for PMNL infiltration, collagen formation, fibroblast numbers and presence of new blood vessels. The combined score for each of these measures were higher in the LM511-E8-treated group from day 14 onwards (overall pathological score in control vs. LM511-E8-treated wound: day 7: 7.7 vs. 8.0; day 14: 12.3 vs. 15.0; day 21: 13.3 vs. 15.7; day 28: 15.0 vs. 17.3).

Qualitative assessment of cytokeratin 10 and 14 were also conducted by immunohistochemistry (Fig. 2). Increased staining of the epithelial cells covering the wound bed in these images indicates greater post-wound re-epithelialization. The LM511-E8-treated group showed higher levels of anti-cytokeratin 14 immunoreactivity from days 7-21 (Fig. 2) and higher levels of anti-cytokeratin 10 than the control group on days 14 and 21 (Fig. 2 control vs. LM511-E8-treated group, for cytokeratin 14: day 7: 0.66 vs. 2.00, day 14: 2.00 vs. 3.66, day 21: 2.66 vs. 4.00 and day 28: 3.33 vs. 4.00. Cytokeratin 10: day 7: 0.66 vs. 2.00, day 14: 1.33 vs. 3.00, day 21: 1.66 vs. 3.33 and day 28: 3.33 vs. 4.00).

Figure 1: LM511-E8 treatment improves the healing rate of second-degree burn wounds. (a) Diagrammatic representation of laminin $\alpha 5 \beta 1 \gamma 1$ (LM511) and LM511-E8 fragment. (b) Representative images of second-degree burn wounds in rats treated daily with 2.5 μ g of LM511-E8 and rats treated with normal saline solution (control). (c) Wound healing plotted as a percentage of the original wound area. Bars represent mean with each point representing one wound. ** $P < 0.01$ and *** $P < 0.001$.

Figure 2: LM511-E8 treatment improves the histology and cytokeratin expression of burn wounds. Rat back-skin burn wounds were treated daily with either normal saline solution (control) or 2.5 μ g of LM511-E8 then euthanized at 3, 7, 14, 21, or 28 days. Tissues were

paraffin-fixed and formalin embedded for histology and immunohistochemistry. (a) Representative images from hematoxylin and eosin-stained sections. (b and c) sections processed for immunohistochemistry with antibodies against cytokeratin 14 (CK14, b) or cytokeratin 10 (CK10, c). Scale bar represents 2000 μm .

Discussion

The results presented in this study demonstrate that the repair process of burn wounds can be improved using a short protein fragment derived from LM511. Specifically, LM511-E8 treatment reduces the wound area rapidly and induces a qualitative improvement in epithelialization, cytokeratin 10 and 14 expression and overall pathological score. Together, these data suggest that the use of LM511-E8 is a promising new treatment for patients with burn injuries.

We believe that these findings will be of clinical relevance for large and slow-to-heal wounds, where provision of an exogenous substrate to promote repair would be most beneficial for recovery. Although we have not investigated its precise mechanism of action, LM511 has been widely studied and the E8 portion in particular contains high-affinity cell surface receptor binding sites, including that for integrin $\alpha 6\beta 1$ and it is likely that the improved wound repair is due to the activation of the signaling pathways that promote epithelialization [27,28]. Moreover, LM511 is usually found in mature endothelial basement membrane and therefore, LM511-E8 fragment could be involved in the maturation of endothelial cells [7,29].

Conclusion

The main question that remains unanswered regards the optimal mode of delivery. Here, we applied LM511-E8 in a drop wise manner to the wounds. While this was effective in these small studies, it would be prohibitively expensive in applying to large wounds in a clinical setting. Integration of LM511-E8 into an artificial substrate should be explored as a treatment option in the future. Further studies to assess the efficacy of LM511-E8 on patients or preclinical models of severe burn wounds and comorbidities such as diabetes.

Conflict of Interest

It is stated that there are no conflicts of interest between the proponents and participants in the present work.

Ethics Section

Animal experiments were approved by the Animal Care and Use Committee of Chulalongkorn University (approval number: 003/2562 on 03/2019).

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Supplementary Data

In-vitro Scratch Closure Assay

Rat Dermal Fibroblasts (RDFs) (Cell Applications, San Diego, CA, USA) were cultured in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37 °C in 95% air and 5% CO₂. The cells were expanded in T-75 Corning™ U-shaped cell culture flasks and harvested at a confluency of approximately 80% using 0.05% Trypsin-EDTA with phenol red (Thermo Fisher Scientific). The cells were then washed with sterile 1× Phosphate-Buffered Saline (PBS) and seeded at 5×10^4 cells/well in 24-well culture plates. The RDFs were grown to confluence and two parallel wounds were made by scraping the cells in the monolayer using a 200 µL pipette tip. Cell debris was removed by washing the wells with 1× PBS and fresh medium supplemented with recombinant LM511-E8 (commercially available as iMatrix-511; Takara Bio Inc., Nippi, Tokyo, Japan) at concentrations of 0, 0.5, 1.0, 2.5 or 5.0 µg/µL was added.

The wounds were photographed under a light microscope at 200× magnification after 0, 12 and 24 h and the wound area was measured using the ImageJ software version 1.52t (National Institutes of Health, Bethesda, MA, USA). All tests were repeated three times and performed in triplicate.

Figure S1: Effects of LM511-E8 dose optimization on rat dermal fibroblast cells in a scratch closure assay. (a) Photographs of the scratch wounds in test and control groups at 0, 12 and 24 h after treatment with 2.5 µg/µL LM511-E8. (b) Box plot of the migration velocities of cells treated with the indicated concentrations of LM511-E8. Boxes represent the values between the 25th and 75th percentile with a line at the median and the upper and lower whiskers represent the range outside the median. *P < 0.05; all tests were repeated three times and performed in triplicate.