Diabetic wound regeneration using peptide-modified hydrogels to target re-epithelialization

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There is a clinical need for new, more effective treatments for chronic wounds in diabetic patients. Lack of epithelial cell migration is a hallmark of nonhealing wounds, and diabetes often involves endothelial dysfunction. Therefore, targeting re-epithelialization, which mainly involves keratinocytes, may improve therapeutic outcomes of current treatments. In this study, we present an integrin-binding prosurvival peptide derived from angiopoietin-1, QHREDGS (glutamine-histidine-arginine-glutamic acid-aspartic acid-glycine-serine), as a therapeutic candidate for diabetic wound treatments by demonstrating its efficacy in promoting the attachment, survival, and collective migration of human primary keratinocytes and the activation of protein kinase B Akt and MAPKp42/44. The QHREDGS peptide, both as a soluble supplement and when immobilized in a substrate, protected keratinocytes against hydrogen peroxide stress in a dose-dependent manner. Collective migration of both normal and diabetic human keratinocytes was promoted on chitosan-collagen films with the immobilized QHREDGS peptide. The clinical relevance was demonstrated further by assessing the chitosan-collagen hydrogel with immobilized QHREDGS in full-thickness excisional wounds in a db/db diabetic mouse model; QHREDGS showed significantly accelerated and enhanced wound closure compared with a clinically approved collagen wound dressing, peptide-free hydrogel, or blank wound controls. The accelerated wound closure resulted primarily from faster re-epithelialization and increased formation of granulation tissue. There were no observable differences in blood vessel density or size within the wound; however, the total number of blood vessels was greater in the peptide-hydrogel-treated wounds. Together, these findings indicate that QHREDGS is a promising candidate for wound-healing interventions that enhance re-epithelialization and the formation of granulation tissue.

diabetic wound healing | peptide | hydrogel | QHREDGS | re-epithelialization

Chronic ulcers are considered a major healthcare challenge because they affect 6.5 million people in the United States (1). Nonhealing wounds, including chronic ulcers, can be caused by a number of common diseases and medications, such as vascular insufficiency, diabetes mellitus, and local-pressure effects, which disrupt the well-orchestrated cellular and molecular interactions during the wound-healing process (2). Specifically, diabetic foot ulcers affect 15% of people with diabetes and are a leading cause of amputation (3). The mechanism underlying diabetic chronic wounds remains elusive, and new interventions for diabetes-impaired wound healing are needed. After almost two decades without new chemical entities approved by the Food and Drug Administration (FDA) (Regranex was approved in 1997), it has been recognized that an optimal wound-healing outcome requires a multifaceted approach that simultaneously addresses various issues (e.g., persistent inflammation, insufficient angiogenesis, and impaired re-epithelialization) (2).

Keratinocytes are the major cell type in the epidermis, the outermost layer of skin. Upon injury, keratinocytes migrate from the wound edge into the wound to re-epithelialize the damaged tissue and restore the epithelial barrier. The hallmark of nonhealing human wounds is nonmigratory and hyperproliferative keratinocytes, resulting in epidermis thickening at the wound edge and an absence of wound closure (4). Scarless embryonic wound healing and complete healing in animals with a high regenerative potential such as newts critically depend on rapid re-epithelialization (5, 6).

Additionally, nonhealing diabetic wounds are trapped in a state of prolonged inflammation characterized by supraphysiologic oxidative stress that can induce keratinocyte injury, dysfunction, and apoptosis (7–9). The supraphysiologic oxidative stress results from the excess production of reactive oxygen species (ROS) by macrophages and neutrophils, coupled with an impaired antioxidant defense capability in response to hyperglycemia (7). Moreover, altered extracellular matrix (ECM) composition in nonhealing wounds and an enhanced ECM degradation rate caused by elevated matrix metalloproteinase levels can impair keratinocyte attachment, leading to aberrant cell signaling and impaired migration (10–12).

To address these challenges, we sought to develop a wound-healing approach that could recapitulate key aspects of scarless embryonic wound healing by (i) promoting effective keratinocyte migration, (ii) protecting the wound-bed cells against oxidative stress, and (iii) providing a new matrix for cell attachment.

Our group has recently described an angiopoietin-1-derived peptide, QHREDGS (glutamine-histidine-arginine-glutamic acid-aspartic acid-glycine-serine), which interacts with integrins, receptors that function in cell adhesion and ECM binding. The QHREDGS peptide, both as a soluble supplement and when immobilized in a substrate, protected keratinocytes against hydrogen peroxide stress in a dose-dependent manner. Collective migration of both normal and diabetic human keratinocytes was promoted on chitosan-collagen films with the immobilized QHREDGS peptide. The clinical relevance was demonstrated further by assessing the chitosan-collagen hydrogel with immobilized QHREDGS in full-thickness excisional wounds in a db/db diabetic mouse model; QHREDGS showed significantly accelerated and enhanced wound closure compared with a clinically approved collagen wound dressing, peptide-free hydrogel, or blank wound controls. The accelerated wound closure resulted primarily from faster re-epithelialization and increased formation of granulation tissue. There were no observable differences in blood vessel density or size within the wound; however, the total number of blood vessels was greater in the peptide-hydrogel-treated wounds. Together, these findings indicate that QHREDGS is a promising candidate for wound-healing interventions that enhance re-epithelialization and the formation of granulation tissue.

The authors declare no conflict of interest.


Significance

Current treatments for diabetic chronic wounds fail to achieve effective therapeutic outcomes. The majority of these treatments focus on angiogenesis, but diabetes often involves endothelial dysfunction. A hallmark of regenerative wound healing is rapid, effective re-epithelialization. In this study, we present QHREDGS (glutamine-histidine-arginine-glutamic acid-aspartic acid-glycine-serine), a prosurvival peptide derived from angiopoietin-1, as a therapeutic candidate that targets re-epithelialization. Immobilized QHREDGS peptide promoted cell survival against hydrogen peroxide stress and collective cell migration of both normal and diabetic human keratinocytes in vitro. The clinical relevance was demonstrated further in type 2 diabetic mice: A single treatment with a low QHREDGS dose immobilized in chitosan-collagen was effective in promoting wound healing, and a single high-dose peptide treatment outperformed a clinically approved porous collagen dressing.


Supporting Information


Supporting Information


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peptide was shown to enhance endothelial cell metabolism, tube-
formation kinetics, and survival in response to apoptotic stimuli (13). OHRGDS also was shown to promote neonatal rat cardiomyocyte
attachment and survival (14), to inhibit human induced pluripotent
stem cell (hiPSC) apoptosis during cell expansion (15), to induce
osteoblast matrix deposition and mineralization (16), and to have
cardiac-protective effects in a chitosan-collagen hydrogel both in
vitro and in vivo (17, 18).

We therefore hypothesized that the OHRGDS peptide could
promote keratinocyte survival and migration and thereby acceler-
ate diabetic wound healing. We investigated the effect of the
OHRGDS peptide as a soluble supplement on the survival of
normal human keratinocytes upon oxidative stress. The effect on
attachment, survival upon oxidative stress, and collective migra-
tion of both normal and diabetic keratinocytes was assessed by
immobilizing the OHRGDS peptide within a chitosan-collagen
film coating. We further investigated the ability of the OHRGDS
peptide to promote diabetic wound repair in vivo using a full-
thickness excision wound model in db/db diabetic mice.

Results

OHRGDS Peptide Prevents H₂O₂-Induced Cell Death in Human Primary Keratinocytes via Akt and MAPKp42/44 Signaling. To evaluate the ef-
effect of the OHRGDS peptide on keratinocytes, we first focused
on normal neonatal human epidermal keratinocytes (HEKs)
cultured with the soluble OHRGDS peptide at doses previously
reported to be effective for endothelial cell survival (low: 100 μM;
high: 650 μM) (13). The percentage of proliferating HEKs in the
population was not affected by the presence of the OHRGDS
peptide at either concentration as quantified by BrdU incorporation
(Fig. 1A). No significant difference in the HEK migration rate was
observed with the soluble OHRGDS peptide at either concentra-
tion (SI Appendix, Fig. S1).

To investigate the effect of the soluble OHRGDS peptide on
keratinocyte survival under oxidative stress, we preconditioned
HEKs by incubating the cells with or without the OHRGDS
peptide and then exposed the HEKs to 500 μM H₂O₂ for 2 h
(Fig. 1B). An endpoint cell integrity assay showed a significant
dose-dependent increase in the percentage of viable HEKs in the
presence of supplemented OHRGDS (Fig. 1C).

Given that the full-length protein from which the OHRGDS
peptide was derived, angiopoietin-1, is known to protect skin cells
from oxidative damage and to increase the activation of the pros-
survival Akt and MAPKp42/44 pathway (19), we investigated whether
improved survival upon H₂O₂ stress in the presence of the
OHRGDS peptide was associated with the up-regulation of Akt and
MAPKp42/44 phosphorylation. HEKs treated with 500 μM H₂O₂
showed transient phosphorylation of both Akt (Fig. 1D) and
MAPKp42/44 (Fig. 1E) at 15 min by Western blot analysis. Indeed, the
presence of the soluble OHRGDS peptide during preconditioning
and H₂O₂ treatment increased the phosphorylation of Akt and
MAPKp42/44, and the increase was dose-dependent (Fig. 1 D and E).

Immovilized OHRGDS Peptide Promotes HEK Attachment, Survival, and Migration in Vitro. Although we observed increased sur-
vival without excessive proliferation in the presence of soluble
OHRGDS, we did not observe enhanced keratinocyte migra-
tion (SI Appendix, Fig. S1). This absence of accelerated migration

Fig. 1. Soluble OHRGDS peptide prevents H₂O₂-
induced cell death in human primary keratinocytes
with up-regulation of Akt and MAPK phosphoryla-
tion. (A) Kct-positive HEKs cultured in the presence
or absence of different concentrations of soluble
OHRGDS peptide did not incorporate significantly
different amounts of BrdU, indicating similar pro-
liferation rates in all three conditions (low peptide:
100 μM; high peptide: 650 μM). (Scale bars: 50 μm.)
(B) HEK survival after H₂O₂ treatment was determined by the EarlyTox
Cell Integrity assay (Molecular Devices). The OHRGDS
peptide protected HEKs against H₂O₂-induced cell
death in a dose-dependent manner. (Scale bars: 200 μm.) One representative experiment is shown;
three independent experiments were performed;
each experiment had nine replicates of each condi-
tion. (D and E) Immunoblots with phosphorylated
Akt or MAPKp42/44 and Akt or MAPKp42/44 antibodies
showed transient activation of Akt and MAPKp42/44
pathways signaling under H₂O₂ stress. GAPDH was
used as a loading control. The presence of the OHRGDS
peptide in the culture medium up-regulated Akt and
MAPKp42/44 phosphorylation. Four independent ex-
periments were performed; each experiment had two
or three independent replicates of each condition.
Data are presented as mean ± SD; *P < 0.05.
motivated our further optimization of the method by which we presented the peptide to the cells. Given that the QHREDGS peptide is reported to function primarily through integrin interactions (13, 15), and an ever-growing body of literature shows that the efficacy of integrin ligands is increased when immobilized to a matrix (20, 21), we covalently immobilized the QHREDGS peptide to a chitosan–collagen hydrogel. Chitosan and collagen interact through a combination of thermal and ionic mechanisms, stabilized by polyanion (collagen) and polycation (chitosan) electrostatic interactions (22). Conjugation of the QHREDGS peptide to chitosan was achieved using previously described methods (23), and chitosan–collagen films with or without immobilized QHREDGS peptide were cast in the wells of 24-well or 96-well plates. Quantification using fluorescently labeled peptide, FITC-QHREDGS, demonstrated effective immobilization in both low (4.7 ± 0.1 nmol/cm²) and high (13.8 ± 1.4 nmol/cm²) peptide concentrations and the absence of the peptide in the control condition (Fig. 2A). Normalized to the mass of chitosan in the films, the amount of immobilized QHREDGS peptide was 14.9 ± 0.3 nmol/mg in the low peptide concentration and 44.1 ± 4.6 nmol/mg in the high peptide concentration.

There was no significant difference in the attachment of HEKs to the various chitosan–collagen films (Fig. 2B). However, in chitosan-only films, in which adhesion was poor, the QHREDGS peptide clearly promoted HEK attachment in a dose-dependent manner (SI Appendix, Fig. S2). This result indicates that, although the QHREDGS peptide can promote HEK attachment, the presence of collagen adhesion sites in the setting of the chitosan–collagen film masks this effect. Furthermore, Western blot analysis showed the increased activation of Akt and MAPKp42/44 during 2-h attachment on chitosan–collagen films in the presence of immobilized QHREDGS peptide (Fig. 2C).

We then investigated the effect of the immobilized QHREDGS peptide on HEK survival following treatment with 500 μM H₂O₂. HEKs were allowed to attach to the chitosan–collagen films for 4 h and then were treated with H₂O₂ for 2 h (Fig. 2D). Subsequent cell-integrity assessment showed an increased percentage of viable HEKs in the presence of the immobilized QHREDGS peptide (Fig. 2E). HEKs treated with 500 μM H₂O₂ and nontreated controls were also compared using Western blot analysis, which showed that the phosphorylation of MAPKp42/44 was increased in the presence of immobilized QHREDGS peptide relative to the control (Fig. 2F).

Keratinocyte migration is essential for wound healing, because a wound cannot heal in the absence of re-epithelialization (24). We therefore assessed the effect of the immobilized QHREDGS peptide on HEK migration in 2D monolayers using an Ibidi migration assay system. Importantly, the Ca²⁺ concentration in the

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**Fig. 2.** Immobilized QHREDGS peptide in chitosan–collagen films promotes the survival and migration of human neonatal primary keratinocytes. (A) Quantification of the amount of QHREDGS peptide immobilized within chitosan–collagen films. n = 3. (B) HEK attachment on the chitosan–collagen films in the presence or absence of conjugated QHREDGS peptide. Image analysis showed no difference in the number of attached HEKs (stained with DAPI) among the three groups. (Scale bars: 200 μm.) Three independent experiments were performed; each experiment had three independent replicates of each condition. (C) Immunoblotting with anti-phosphorylated Akt or MAPKp42/44 and anti-Akt or MAPKp42/44 showed up-regulation of MAPKp42/44 and Akt activation during HEK attachment. GAPDH was used as a loading control. Three independent experiments were performed; each experiment had two independent replicates of each condition. (D) Experimental timeline. Cells were harvested for attachment Western blotting 2 h after seeding. H₂O₂ was applied 4 h after seeding, and the cells were harvested 15 min later for Western blotting. Live/dead staining was performed after 2 h of H₂O₂ treatment. (E) HEK survival after H₂O₂ treatment was determined by the EarlyTox Cell Integrity assay. QHREDGS peptide in the chitosan–collagen film protected HEKs against H₂O₂-induced cell death in a dose-dependent manner. One representative experiment is shown; three independent experiments were performed; each experiment had four independent replicates of each condition. (F) Immunoblotting showed up-regulation of the MAPKp42/44 and Akt phosphorylation in HEKs under H₂O₂ stress at 15 min. GAPDH was used as a loading control. Three independent experiments were performed; each experiment had two independent replicates of each condition. (G) Representative examples of HEK-wounding experiments on chitosan–collagen films in the presence or absence of conjugated QHREDGS peptide. (Scale bars: 200 μm.) HEK migration on the QHREDGS-immobilized films was accelerated compared with HEK migration on the control peptide-free films. One representative experiment is shown; three independent experiments were performed; each experiment had six independent replicates of each condition. Data are presented as mean ± SD; *P < 0.05.
culture medium was increased from 0.06 mM to 0.12 mM upon initiation of the migration assay to ensure collective HEK migration (essential for wound healing), as demonstrated by the formation of E-cadherin-mediated cell–cell junctions (SI Appendix, Fig. S34). The presence of the immobilized QHREDGS peptide accelerated collective HEK migration in a dose-dependent manner (Fig. 2G). The accelerated migration was not caused by increased proliferation, because there was no difference in cell density among the three groups as characterized at the migration endpoint (SI Appendix, Fig. S3B).

**Immobilized QHREDGS Peptide Promotes Diabetic Human Primary Keratinocytes’ Attachment, Survival, and Migration in Vitro.** In diabetic chronic wounds, keratinocytes experience hyperglycemia and supraphysiological oxidative stress, which challenge keratinocyte’s proliferation and survival (7, 25). Therefore, we examined the effect of the immobilized QHREDGS peptide on adult diabetic human epidermal keratinocytes (DHEKs) by seeding DHEKs onto chitosan–collagen films in the presence or absence of immobilized QHREDGS peptide. Similar to the results with normal HEK cells, we found the presence of the immobilized QHREDGS peptide promoted DHEK attachment to chitosan-only films (SI Appendix, Fig. S3A).

Representative immunoblots of phosphorylated and total MAPK 

Fig. 3. Immobilized QHREDGS peptide in chitosan–collagen films promotes the survival and migration of adult DHEKs. (A) DHEK attachment on the chitosan–collagen films in the presence or absence of immobilized QHREDGS peptide. Image analysis showed no difference in the number of attached DHEKs (stained with TO-PRO) among the three groups. (Scale bars: 200 μm.) Three independent experiments were performed; each experiment had three independent replicates of each condition. (B) Immunoblotting showed up-regulation of MAPK 

Fig. 4. Western blot analysis showed that the activation of MAPK and Akt was increased in DHEKs in the presence of the QHREDGS peptide during a 2-h attachment (Fig. 3B).

Because of prolonged inflammation (25), human chronic wounds experience three to four times higher oxidative stress (26) and oxidative damage (27) than acute wounds. To mimic this scenario, we investigated the effect of immobilized QHREDGS peptide on DHEK survival following a 2-h treatment with 2 mM H$_2$O$_2$ (an exposure four times higher than used for the HEK survival assay) after DHEKs were allowed to attach to the chitosan–collagen films for 4 h (Fig. 3C). Cell-integrity assessment showed that DHEK survival under H$_2$O$_2$ stress was improved in the presence of immobilized QHREDGS peptide (Fig. 3D), despite the higher H$_2$O$_2$ concentration used. DHEKs treated with 2 mM H$_2$O$_2$ and nontreated controls were also compared by Western blot analysis, which showed that the phosphorylation of Akt and MAPK$_{p42/44}$ upon H$_2$O$_2$ treatment was increased in the presence of immobilized QHREDGS peptide in a dose-dependent manner (Fig. 3E).

We then assessed the effect of immobilized QHREDGS peptide on DHEK migration using the Ibidi migration assay. Importantly, the Ca$^{2+}$ concentration in keratinocyte growth medium (KGM),
A low dose of QHREDGS peptide immobilized to chitosan-collagen hydrogel is sufficient to promote wound healing in db/db diabetic mice. (A) Representative image of the 8-mm full-thickness dorsal wounds on db/db diabetic mice. (B) Representative gross images of the initial wounds on day 0 (D0) and the wounds at 14 d (D14) after treatment with no hydrogel (Blank), with peptide-free chitosan-collagen hydrogel (Ctrl), or with a low dose of QHREDGS peptide conjugated to chitosan-collagen hydrogel (Low peptide). Quantification of the wound size as a percentage of the original wound area revealed faster wound closure in the peptide-treated mice at days 8–14, n = 4. (C) Representative images of Trichrome-stained tissue sections of wounds treated with no hydrogel, with peptide-free chitosan-collagen hydrogel, or with a low dose of QHREDGS peptide conjugated to chitosan-collagen hydrogel on day 14. Black arrowheads indicate wound edges; red arrowheads indicate the tips of the healing epithelial tongue. (Scale bar: 3 mm.) (Insets) The tips of the healing epithelial tongue were confirmed by pan-keratin staining. [Low and High Magnification images from slide scanned image at 20x using the Aperio ScanScope XT (Aperio Technologies, USA).] (D) Quantification of wound size from histological samples collected 14 d after treatment. (i) Image analysis showed no significant difference in the wound-edge distance among the three groups. (ii) The low-peptide treatment significantly reduced the size of epithelial gap, indicating accelerated wound closure compared with the blank and control groups. (iii) The low-peptide treatment significantly increased the re-epithelialization percentage at the end of experiment compared with the blank and control groups. (iv) The low-peptide treatment significantly increased the size of the granulation tissue compared with the blank and control groups. (v) Average thickness of the epidermis within 300 μm of the leading edge of the wound. The epidermal thickness in the low-peptide group was lower than in the blank and control groups. n = 4. Data are presented as mean ± SD; *P < 0.05.

0.1 mM, was sufficient to ensure collective DHEK migration, as demonstrated by the formation of E-cadherin–mediated cell–cell junctions, without additionally elevating the Ca²⁺ concentration (SI Appendix, Fig. S5A). The presence of the immobilized QHREDGS peptide also accelerated DHEK collective migration (Fig. 3F), and this acceleration was not caused by differences in cell density as characterized at the migration end point (SI Appendix, Fig. S5B).

QHREDGS-Immobilized Hydrogel Promotes Wound Healing in db/db Diabetic Mice. We investigated whether the QHREDGS peptide immobilized to the chitosan-collagen hydrogel could accelerate wound healing in diabetic mice. This hydrogel system was chosen as a delivery vehicle because of its rapid gelation under physiological conditions and its persistence for a period of 3 wk in vivo (18). In vivo biocompatibility had been demonstrated in previous myocardial infarction model studies (18). Therefore, only one application of the hydrogel onto the wounds was needed for the 2-wk study. A full-thickness excision wound (Fig. 4A) was created on 8-wk-old male BKS.Cg-Dock7<sup>+/−</sup> Lepr<sup>db</sup>/+ mice (db/db). This model was selected because the animal is leptin-receptor deficient and represents a type II diabetes model characterized by hyperglycemia, obesity, hyperinsulinemia, and impaired wound healing. Moreover, this strain heals wounds primarily by the formation of granulation tissue rather than by contraction (28).

Quantification using fluorescently labeled peptide demonstrated that in reconstituted chitosan solutions the amount of conjugated QHREDGS peptide was 17.5 ± 2.2 nmol/mg chitosan in low-concentration conditions and 41.5 ± 1.4 nmol/mg chitosan in high-concentration conditions. In the final chitosan–collagen hydrogel these concentrations were converted to a peptide concentration of 43.8 ± 4.4 μM in the low-concentration conditions and 103.8 ± 3.5 μM in the high-concentration conditions. With a view to future clinical translation, we first tested the low-concentration condition in the in vivo studies to minimize the amount of peptide applied to the wound. A single application of 50 μL low-concentration chitosan–collagen hydrogel (2.2 nmol immobilized QHREDGS peptide; low-peptide treatment) was applied to the wound. The chitosan–collagen hydrogel alone without the peptide (control treatment) and a no hydrogel/no peptide (blank condition) were used as controls. A secondary dressing, Tegaderm film, was applied on top of the wound, with or without the hydrogel, to maintain a moist environment. As shown in Fig. 4B, the presence of immobilized QHREDGS in the hydrogel resulted in significantly smaller wounds on day 14 than in the controls. Image analysis of the wound gross morphology performed by an investigator blinded to the...
study groups demonstrated faster wound healing in the low-peptide group starting on day 8. Administration of the chitosan-collagen hydrogel without the immobilized OHREDGS peptide (control treatment) had no significant effect on the wound closure rate compared with the blank controls.

We also examined the wound histology by Masson’s trichrome staining and confirmed the location of the epithelial tongue using pan-keratin staining (Fig. 4C). The wound edge was defined as the distance between the two boundaries of intact skin (thin musculature of the panniculus carnosus). There was no significant difference among the three groups in the wound-edge distance, indicating no difference in wound contraction over 14 d (Fig. 4D, i). The epithelial gap, defined as the distance between the two advancing epithelial tongues (Fig. 4C), was smaller in the low-peptide group than in the blank and control groups (Fig. 4D, ii and SI Appendix, Fig. S6). The re-epithelialization percentage, defined as the ratio of the distance that has been re-epithelialized over the wound-edge distance, was significantly higher in the presence of the OHREDGS peptide than in the controls (Fig. 4D, iii and SI Appendix, Fig. S6). The low-peptide group also developed significantly more granulation tissue (Fig. 4D, iv) than did the controls. Moreover, the epidermal thickness of the advancing epithelial tongues was significantly smaller in the peptide group than in the blank and control groups (Fig. 4D, v). There was no difference in the epithelial thickness of the skin remnant from the wounds among the three experimental groups (SI Appendix, Figs. S8 A and C, i–iii) and smooth muscle actin (SMA) (SI Appendix, Figs. S8 B and C, iv) indicated no significant differences in the blood vessel density (SI Appendix, Figs. S8 C, ii, and S9) and myofibroblast content among the groups (Fig. 4D, iii), although total number of blood vessels in the wound was significantly higher in the low-peptide group than in the blank and control groups at day 14 (SI Appendix, SI Results and Fig. S8 C, iii).

**High Peptide Hydrogel Outperforms a Clinically Approved Wound Dressing in Wound Closure of db/db Diabetic Mice.** To demonstrate the full potential of the peptide-modified hydrogels and as a benchmark against an FDA-approved and clinically available treatment, a high-peptide hydrogel (high-peptide treatment) was compared with CoActive collagen dressing (collagen treatment) in vivo. After 21 d, visual inspection indicated essentially closed wounds in animals in the high-peptide group (Fig. 5A). When the animals were killed, analysis of wound images indicated 100% wound closure in three of the five animals in the high-peptide group, in one of five animals in the collagen-treatment group, and in none of the five animals in the blank or the peptide-free hydrogel controls. The advantages of the high-dose peptide hydrogel treatment became apparent at day 8 after treatment (Fig. 5B) and persisted throughout the healing period up to day 21, at which point the wounds in the high-peptide group were essentially closed. In the linear range of wound closure (Fig. 5B, days 2–16), the fitted line slopes indicated a significantly higher rate of wound closure in the high-peptide group (8% of wound area/d) than in the blank (3%/d) and peptide-free hydrogel (4%/d) control groups and in collagen-treatment (5%/d) groups (SI Appendix, Fig. S10). There was no significant difference in the rate of wound closure in the peptide-free hydrogel and the CoActive dressing groups; the rate of wound closure was significantly faster in the high-peptide hydrogel group than in all other groups (SI Appendix, Fig. S10).

Histological analysis (Fig. 5C) indicated that only the animals in the high-peptide group had a significant difference in the wound-edge distance (Fig. 5D, i), epithelial gap (Fig. 5D, ii), re-epithelialization percentage (Fig. 5D, iii), and epithelial thickness (Fig. 5D, iv) relative to the blank group. In the wounds in the high-peptide group the epithelial thickness was also comparable to that of the unwounded epidermis (Fig. 5D, iv and SI Appendix, Fig. S7B), whereas the other treatment groups had epidermis that was significantly (more than two times) thicker than the unwounded epidermis. According to these histological measures of wound closure and epidermis quality, the high-peptide group significantly outperformed both the control group treated with peptide-free hydrogel and the collagen treatment group (Fig. 5D, ii–iv). Notably, in the high-peptide group, one mouse with complete wound closure also exhibited hair regrowth at the wound periphery as seen in gross morphology (SI Appendix, Fig. S114) and histology (SI Appendix, Fig. S11B) images. Picrosirius red staining indicated disruption of the native collagen organization in day 21 wounds in blank-, peptide-free hydrogel-, and collagen-treated mice (SI Appendix, Fig. S12). In contrast, collagen organization equivalent to the unwounded skin was identified in wounds treated with the high-peptide immobilized hydrogel (SI Appendix, Fig. S12). Staining for the neural marker protein gene product 9.5 (PgP9.5) confirmed a low density of positive cells in all groups, with no appreciable differences among groups (SI Appendix, Fig. S13). This result is consistent with the inability of diabetic mice to regenerate neurons fully (29–31). There were no significant differences in the vascular density among the groups at day 21 (SI Appendix, Fig. S14).

To delineate further whether there were differences in blood vessel densities in the early stages of healing, we compared the time course of early vessel development in the high-peptide and blank groups. At days 4 and 8 after injury there were no appreciable differences in blood vessel density in the two groups (SI Appendix, Fig. S15). We did, however, observe an increase in vessel density and in CD31+ area between day 4 and day 8 in the high-peptide group but not in the blank control group (SI Appendix, Fig. S15). Blood vessel density and the percentage of area covered by endothelial cells remained statistically unchanged between day 8 (SI Appendix, Fig. S15) and day 21 (SI Appendix, Fig. S14) according to ANOVA analysis. Collectively these results indicate that the treatment with high-dose peptide hydrogel leads to sufficient angiogenesis to drive the rapid formation of granulation tissue without the massive vessel overgrowth early on followed by a pronounced vessel regression that is often reported with treatments that overstimulate early angiogenesis (32–34).

**Discussion**

Angiogenesis has been a primary target of therapeutic interventions in wound healing by local application of growth factors [e.g., epidermal growth factor (35, 36), fibroblast growth factor (37, 38), vascular endothelial growth factor (39, 40), and angiopoietin (41)] or angiogenic cells (42, 43). However, diabetic patients are often reported to have dysfunctional endothelium that cannot respond efficiently to growth-factor stimulation (44, 45). Diabetics also suffer from the insufficient recruitment of circulating endothelial progenitor cells, which are critical for wound repair (46, 47). As a result, many of these approaches failed to meet the FDA-accepted primary efficacy endpoint standard of “complete wound closure within 12 weeks for any dose” (48).

We chose to take a different approach here, by considering the hallmarks of true regenerative healing as observed during the closure of embryonic wounds and scarless healing in model organisms such as flies, zebrafish, and newts. In all these models, rapid, coordinated, and collective migration of epidermal cells is critical for regenerative healing (5, 6). Hence, we focused on developing a hydrogel treatment that would promote the collective migration of keratinocytes and enhanced formation of granulation tissue. Different mechanisms have been proposed to explain the cell–cell interactions in collective cell migration, including local tractions pulling cooperatively toward unfilled space (termed “kenotaxis”) (49), mechanical exclusion interactions between cells (50), and intercellular adhesion and tension (51). In our in vitro studies, calcium concentration was increased when necessary to ensure that both normal and diabetic keratinocytes migrated collectively rather than individually so that...
the treatment effect on collective migration could be described accurately.

Following its discovery (52), there has been a growing recognition that angiopoietin-1 is an important regulator of cellular processes including vascular protection (53), cardiac remodeling (54–56), inflammation (57–59), and wound healing (41). Because of the insolubility of angiopoietin-1, its derivatives have been developed to promote wound healing by angiogenesis (60–62). Here, we assessed the effect of a water-soluble peptide, OHREDGS, derived from the fibrinogen-like domain of angiopoietin-1 and conserved among species (54), on promoting the survival and collective migration of normal and diabetic keratinocytes. In this study, supplementation of the culture medium with soluble OHREDGS peptide protected human keratinocytes against H2O2 stress and induced up-regulation of Akt and MAPKp42/44 phosphorylation. These findings are consistent with previous reports for angiopoietin-1 in human keratinocytes and melanocytes (19). Here we demonstrate the effect of the OHREDGS peptide on cell migration and cutaneous wound healing. Our previous studies also reported that a scrambled peptide (DGQESH or DQSHGER) does not exert prosurvival effects similar to that of the OHREDGS peptide in cardiac cells (14, 63), endothelial cells (13), and induced pluripotent stem cells (15), motivating the omission of the scrambled peptide in the studies described here.

Importantly, the OHREDGS peptide functions through interactions with β1-containing integrins that are involved in cell–matrix interactions, rather than Tie2 receptors that reside mainly on endothelial cells (13, 15, 18). Thus the OHREDGS peptide can act on various cell types, including keratinocytes, that express the αβ1-integrin implicated in their enhanced survival and migration (64–66). The integrin interaction also motivated the presentation of the OHREDGS peptide as a matrix-bound ligand. Furthermore, the short peptide sequence OHREDGS can be modified using versatile chemical methods and does not require a specific orientation or conformation to function, as does the full-length protein (67), and peptides are less susceptible than full-length proteins to degradation caused by proteolysis or hydrolysis during modification and after delivery to the native environment (68).

Here, we used 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) chemistry to conjugate the OHREDGS peptide to the backbone of chitosan, which is a zero-length cross-linker that does not add any other moieties to the final product; these features are important for further clinical translation. Peptide immobilization also provides the advantages of localized action in the target tissue and a lower total dose than required for application in an

![Fig. 5. A high dose of OHREDGS peptide immobilized to chitosan-collagen hydrogel outperforms an approved wound-healing dressing in db/db diabetic mice. (A) Representative gross images of the initial wounds on day 0 (D0) and day 21 (D21) after treatment with no hydrogel (Blank), with peptide-free chitosan–collagen hydrogel (Ctrl), with ColActive collagen dressing (Collagen), or with a high dose of OHREDGS peptide conjugated to chitosan–collagen hydrogel (High Peptide). (B) Quantification of the wound size as a percentage of the original wound area revealed a faster wound-closure rate in the peptide-treated mice. n = 5 per group. (C) Representative images of Trichrome-stained tissue sections on day 21. Black arrowheads indicate wound edges; red arrowheads indicate the tips of the healing epithelial tongue. [Low magnification images from slide scanned image at 20x using the Aperio ScanScope XT (Aperio Technologies, USA). (Scale bar: 3 mm.) (D) Quantification of wound size from histological samples collected 21 d after treatment. (i) The high-peptide treatment significantly reduced the wound-edge distance compared with the blank group. (ii) The high-peptide treatment significantly reduced the size of epithelial gap compared with the other three groups, indicating accelerated wound closure. (iii) The high-peptide treatment significantly increased the re-epithelialization percentage compared with the other three groups. (iv) The high-peptide treatment significantly decreased the epithelial thickness compared with the other groups, to a level comparable to undamaged epithelium. n = 4 or 5 per group. Data are presented as mean ± SD; *P < 0.05.](image-url)
encapsulated or soluble form. We confirmed that the prosurvival effect of the OHREDGS peptide was preserved after it was conjugated and immobilized in the chitosan–collagen films and that the immobilized OHREDGS peptide was able to promote keratinocyte collective migration, whereas the soluble peptide did not. Notably, in the context of the chitosan–collagen film system, the conjugated OHREDGS peptide is presented in close proximity to collagen, thereby creating an environment reminiscent of the collagen–glycosaminoglycan interaction in the native ECM (69).

Using DHEKs from an adult diabetic patient, we determined that the prosurvival, promigratory effects of immobilized OHREDGS peptide could be translated to diabetic keratinocytes. Diabetic chronic wounds often occur in the elderly population (the source of the primary DHEKs in this study) because of age-associated comorbidities (70), and advanced protein glycosylation that can alter the functional properties of important ECM components, such as collagen, has been associated with diabetes and aging (71). Despite these challenges, the immobilized OHREDGS peptide was effective in the in vitro diabetic wound-healing model, suggesting the possibility of in vivo benefits for diabetic wound healing.

Although our previous in vitro study showed that the OHREDGS peptide promoted endothelial cell survival, metabolism, and tube formation mediated by integrin interactions (13), the accelerated diabetic wound healing shown in the animal studies here was not associated with the significantly enhanced blood vessel density per area of granulation tissue. In the peptide-treated group there was more granulation tissue and consequently a higher total number of blood vessels in these wounds, but there was no increase in blood vessel density relative to the other groups. This finding is consistent with our previous study in a rat myocardial infarction model, which showed an increase in large vessels within the myocardial infarction border zone but no difference in the total vascularization (18). Angiogenesis is critically important for wound healing (72). We view the stable and steady growth of blood vessels in our system as an advantage, because previous studies reported leaky vasculature and, in some cases, angionoma formation in treatments that overemphasized angiogenesis (73).

Alternatively, the diabetic endothelium is associated with enhanced degradation of nitric oxide, an important regulator of inflammation, angiogenesis, and re-epithelialization, because of the presence of excessive ROS (74). In previous studies, the OHREDGS peptide could induce enhanced endothelial cell nitric oxide production (13). Hence, it is possible that the improved wound-healing effects induced by the OHREDGS peptide might be attributable in part to nitric oxide production. In future studies, the OHREDGS peptide could be combined with biomaterials (72) and/or peptides (62) shown to strongly enhance angiogenesis in wound healing to investigate synergistic effects.

Our previous study demonstrated the retention of the chitosan–collagen hydrogel over 3 wk postinjection in the infarcted rat heart, an extremely dynamic, inflammatory environment (18). In this study, we applied the OHREDGS peptide in the same chitosan–collagen hydrogel as a single treatment and found that even a low dose was sufficient to accelerate wound healing in a clinically relevant, genetically modified db/db diabetic mouse model. Moreover, a single application of a high peptide dose could induce wound healing, we selected a larger, 8-mm diameter initial wound size rather than the commonly used 6-mm wound (76, 79). A low dose of peptide was tested in vivo based on the concept that pharmaceutical agents should be applied at a minimal effective dosage to avoid potential side effects. An increased rate of mortality secondary to malignancy was reported for patients treated with >45 g of Regranex (180 nmol PDGF-BB) (89). A high dose was tested in vivo because of the more profound effects on keratinocytes observed with the high peptide dose in vitro. Here, a single low dose of 4.4 nmol peptide/cm² of wound area was effective in promoting granulation tissue formation and wound closure in vivo, whereas daily application of 0.4 nmol Regranex/cm² of wound area was needed to promote granulation tissue formation but did not shorten the time to wound closure in a db/db mouse model (90).

The OHREDGS peptide is available by cost-effective synthesis with a precisely defined composition, offering an additional advantage in potential clinical applications. The cost of the synthetic OHREDGS peptide used in our study was $1.6 Canadian dollars (CAD) per mg, which is ~20,000 times cheaper than the commercially available rhPDGF-BB, an active component of the FDA-approved Regranex, which costs $33,000 CAD per mg (Fisher Scientific). The choice of the hydrogel components, collagen and chitosan, was motivated by clinical translation considerations. Chitosan has been approved by the FDA for use in humans in topical applications (e.g., HemCon bandages). Similarly, collagen is a component of numerous wound dressings currently on the market in different formulations such as freeze-dried sheets, pastes, pads, powders, and gels (e.g., INTEGRA Matrix Wound Dressing, BIOSTEP collagen matrix dressing, BGC Matrix, Stimulon Collagen, ColActive, and others).

Here, we have demonstrated that in diabetic wounds keratinocyte survival and collective migration represent promising alternative therapeutic targets that support re-epithelialization, a hallmark of wound regeneration and closure. In a genetically modified diabetic mouse, the immobilized OHREDGS peptide accelerated the wound-healing process by promoting the rate of re-epithelialization and formation of granulation tissue without significantly affecting angiogenesis. Moreover, the immobilized OHREDGS peptide did not increase the number of α-SMA–positive cells (e.g., myofibroblasts) that can induce the antiangiogenic wound-regeneration processes of wound contraction, collagen overproduction, and scar formation (91).

Our reported results give rise to a number of questions. Mainly, the efficacy of a potential therapeutic intervention providing synergistic regulation of angiogenesis and re-epithelialization in diabetic chronic wounds is yet unknown. Notably, the chitosan–collagen hydrogel system is capable of simultaneously delivering
other molecules, because the chitosan backbone is amenable to various chemical modifications (92, 93).

In conclusion, the QHRQDS peptide promoted keratinocyte adhesion and collective migration in vitro, as well as survival against H2O2 stress through Akt and MAPK pathways. In vivo, the QHRQDS peptide immobilized to a chitosan-collagen hydrogel accelerated diabetic wound healing by enhanced re-epithelialization and granulation tissue formation. Together, our data in both normal and diabetic human primary keratinocytes and in a db/db diabetic mouse model demonstrate the translational relevance of the QHRQDS peptide in treating diabetic wounds. We propose the QHRQDS peptide as a therapeutic candidate for promoting diabetic wound healing.

Materials and Methods

Study Design. We hypothesized that the QHRQDS peptide would promote keratinocyte migration and survival under H2O2 stress and that a single application of QHRQDS-immobilized chitosan-collagen hydrogel would accelerate wound healing in diabetic mice. Endpoints and peptide dosage were selected on the basis of previous studies and preliminary results (18). For animal studies, mice were randomized, and gross morphology analysis was performed by an investigator blinded to the study groups.

Details on primary human keratinocytes cell culture, evaluation of soluble QHRQDS in vitro, the proliferation assay, H2O2 treatment of HEKs with soluble QHRQDS peptide, the conjugation of QHRQDS to chitosan, solvent casting of chitosan-film films, coating validation; keratinocyte attachment on chitosan-only films, H2O2 treatment of keratinocyte on the chitosan–collagen films, the EarlyTox cell-integrity assay, Western blotting, the migration assay, calcium increase for HEK migration, immunostaining, histological analysis, and microvesSEL and positive staining analysis algorithms are given in SI Appendix, SI Materials and Methods.

Animals, Wound Model, and Treatment. The Animal Care Committee of the University of Toronto approved all described animal studies. Eight-week-old, genetically diabetic, maleBKS-Cg-Dock7R1/2 Lepmgj1 mice (db/db) (Stock 000642) were ordered from Jackson Laboratories. Mice were acclimatized for 1 wk, and their blood glucose levels were tested with a glucometer (Accu-Chek; Aviva) to confirm that plasma glucose levels were above 300 mg/dL on the day before surgery.

The chitosan–collagen hydrogel was prepared in a manner similar to that described previously (23). The final hydrogel consisted of 2.5 mg/mL chitosan (with or without conjugated QHRQDS peptide) and 2.5 mg/mL type I collagen neutralized by 1 N NaOH and 10x PBS. The final hydrogel solution was mixed and kept on ice until use. For in vivo application, the pre-gel solution was warmed for about 10 min at 37 °C to initiate the gelling process and was applied to the wound site with a 2.35-gauge needle. To benchmark the peptide hydrogel versus an FDA-approved therapy, a commercially available ColActive collagen dressing was gifted by Covalon Technologies, Inc.

Mice were anesthetized with inhaled isoflurane (5%), and the dorsal surface of the mouse was shaved with an electric shaver, followed by treatment with a hair-removal cream (Veet). Betadine and 70% ethanol were applied in series to the surgical site. Eight-millimeter biopsy punches (WVR) were used to create middorsal full-thickness wounds by excising the epidermis and dermis, including the panniculus carnosus. Fifty microliters of hydrogel containing a total of 2.2 nmol peptide (low-peptide group), 50 μL of hydrogel containing a total of 5.5 nmol peptide (medium-peptide group), or a circular 2.5 mm in diameter of ColActive collagen dressing (collagen group) was applied topically to the wound beds, or the wounds were left untreated (blank group). The wound beds then were covered by Tegaderm film (Figs. 4B and 5A). Buprenorphine (0.03 mg/kg) was given s.c. before and immediately after the surgery as an analgesic. Thereafter, the mice were housed individually and observed every other day. Digital photographs of wounds were taken every 2 d at the same distance by a camera (Canon) with a calibration scale on the side. Mice were killed using CO2 asphyxiation, followed by cervical dislocation, on day 4 (blank and high-peptide groups), day 8 (blank and high-peptide groups), day 14 (blank, control, and low-peptide groups), or day 21 (blank, control, collagen, and high-peptide groups) as indicated in the Results and figures.

Statistical Analysis. All results are presented as mean ± SD. Statistical analysis was performed using SigmaPlot 11.0. Differences between experimental groups were analyzed using one-way or two-way ANOVA, followed by a Tukey post hoc test for pairwise comparison. Linear regression was used to calculate wound-closure rates, and significant differences were determined by comparing the slopes of the fitted lines. A value of P < 0.05 was considered statistically significant.

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