A Small Chimeric Fibronectin Fragment Accelerates Dermal Wound Repair in Diabetic Mice

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Objective: During wound repair, soluble fibronectin is converted into biologically active, insoluble fibrils via a cell-mediated process. This fibrillar, extracellular matrix (ECM) form of fibronectin stimulates cell processes critical to tissue repair. Nonhealing wounds show reduced levels of ECM fibronectin fibrils. The objective of this study was to produce a small, recombinant wound supplement with the biological activity of insoluble fibronectin fibrils.

Approach: A chimeric fibronectin fragment was produced by inserting the integrin-binding Arg-Gly-Asp (RGD) loop from the tenth type III repeat of fibronectin (FNIII10) into the analogous site within the heparin-binding, bioactive fragment of the first type III repeat (FNIII1H). FNIII1H^{RGD} was tested for its ability to support cell functions necessary for wound healing, and then evaluated for its capacity to accelerate healing of full-thickness dermal wounds in diabetic mice.

Results: *In vitro*, FNIII1H^{RGD} supported cell adhesion, proliferation, and ECM fibronectin deposition. Application of FNIII1H^{RGD} to dermal wounds of diabetic mice significantly enhanced wound closure compared with controls (73.9% ± 4.1% vs. 58.1% ± 4.7% closure on day 9, respectively), and significantly increased granulation tissue thickness (2.88 ± 0.75-fold increase over controls on day 14).

Innovation: Recombinant proteins designed to functionally mimic the ECM form of fibronectin provide a novel therapeutic approach to circumvent diminished fibronectin fibril formation by delivering ECM fibronectin signals in a soluble form to chronic wounds.

Conclusion: A small, chimeric fibronectin protein was developed. FNIII1H^{RGD} demonstrated enhanced bioactivity *in vitro* and stimulated wound repair in a murine model of chronic wounds.

Keywords: fibronectin, extracellular matrix, wound healing, diabetes

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Submitted for publication June 16, 2015.
Accepted in revised form September 23, 2015.

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INTRODUCTION

CHRONIC WOUNDS, INCLUDING diabetic, venous, and pressure ulcers, impose a significant healthcare burden worldwide.¹ Approximately 50 million people, including nearly 7 million Americans, suffer from chronic wounds.^{1,2} These wounds cause pain and discomfort for the patient, with health complications ranging from serious infections to limb amputations. Diabetes, obesity,

and aging are major underlying causes of chronic wounds. The prevalence of diabetic foot ulcers is between 2% and 4%, with a lifetime risk of ~25%.^{3–5} Pressure ulcers develop frequently in immobile and debilitated patients, with an overall prevalence of ~15% in both acute and long-term care facilities.⁶ Leg ulcers arising from circulatory problems affect even more individuals and have a recurrence rate as high as 70%.⁷

Currently, 12% of adults in the United States are diabetic, while 35% are obese.⁸ Furthermore, the number of adults aged 65 and older is expected to grow from 35 to 72 million in 2030, representing ~20% of the United States population.⁹ The increasing prevalence of chronic and hard-to-heal wounds has led to annual global expenses of more than US\$9 billion.² Managing nonhealing diabetic ulcers is difficult and requires a multifaceted approach for treatment.¹⁰ Standard treatment approaches include surgical debridement followed by moist dressings and infection control.^{5,11} Newer therapies include topical antimicrobials, oxygen or other gases, bioengineered skin substitutes, compression or negative pressure therapy, skin grafts, and application of platelets, stem cells, and recombinant growth factors.^{10,12} However, a recent review of the literature by the International Working Group of the Diabetic Foot¹³ was unable to identify clear, robust effects of any of the newer therapies in well-controlled clinical trials.

Wound repair requires complex and coordinated interactions between cells and extracellular matrix (ECM) molecules.¹⁴ In healthy individuals, wound healing progresses quickly, with minimal scar formation and the return of normal tissue structure and function. In contrast, wounds that do not heal in a timely manner or that do not possess sufficient mechanical strength are considered chronic.¹⁰ Fibronectin is a principal component of the ECM and plays a key role in tissue development.¹⁵ Unlike collagens and laminins, soluble fibronectin molecules are assembled into the ECM as dense, insoluble fibrils via a cell-dependent, tightly regulated process.¹⁶ Soluble fibronectin molecules are nonreactive with adhesion receptors, whereas the interaction of cells with ECM fibronectin fibrils stimulates a variety of cell and tissue functions. Fibronectin matrix assembly is normally rapidly upregulated following tissue injury, while reduced fibronectin matrix deposition is associated with abnormal wound repair and chronic wounds.¹⁷ Given the role of ECM fibronectin in orchestrating ECM organization¹⁸ and in regulating cell behaviors that are critical for normal tissue repair,^{19–21} defective or reduced fibronectin matrix assembly by dysfunctional cells likely has profound effects on the ability of tissues to heal. Thus, therapeutic approaches that circumvent diminished fibronectin matrix assembly by providing injured cells with synthetic fibronectin matrix analogs may provide a new treatment approach to “jump start” healing in chronic wounds.

Many of the biological activities of fibrillar fibronectin are mediated by a matricryptic, heparin-

binding site located within the first type III repeat of fibronectin (FNIII1).^{22,23} Conformational changes introduced to FNIII1 during fibril formation or in response to compressive, tensile or shear forces, serve to expose this normally cryptic site and initiate cell and tissue responses, including cell proliferation, epithelial cell migration, contractility, and vasodilation.^{19–21,24} To deliver ECM fibronectin signals to cells directly, we developed a series of soluble fibronectin fragments, engineered to mimic the ECM conformation of FNIII1. To do so, the “open”, heparin-binding fragment of FNIII1 (FNIII1H) was coupled directly to variants of the integrin-binding domain (FNIII8–10).^{25,26} This strategy was designed to bypass the need for injured and/or poorly functioning cells to assemble insoluble ECM fibrils within the wound space. We showed previously that different fibronectin matrix mimetics, produced using variants of the cell-binding domain (FNIII1H,8–10, FNIII1H,8,10, and FNIII1H,8^{RGD}), enhanced cell growth, migration, and contractility to a similar or greater extent than full-length fibronectin.²⁶ Moreover, all three recombinant proteins accelerated healing of full-thickness skin wounds in a mouse model of diabetes mellitus type I, suggesting that the full integrin-binding domain (*i.e.*, FNIII8–10) was not required to accelerate wound repair. Thus, in this study, a smaller recombinant chimeric fragment was produced, in which the minimal, integrin-binding RGD loop²⁷ of FNIII10 was inserted directly into an analogous site within the bioactive FNIII1H fragment. FNIII1H^{RGD} was tested for its ability to support cell functions necessary for wound healing, and then evaluated for its capacity to accelerate the healing of full-thickness dermal wounds in diabetic mice.

CLINICAL PROBLEM ADDRESSED

Chronic wounds affect the lives of millions of people worldwide, and are a growing financial burden on our healthcare system. New treatment methods are urgently needed to rapidly close chronic wounds to decrease the risk of infection, prevent fluid loss, and promote the natural healing process. We envision the clinical use of soluble fibronectin matrix mimetics as a new approach to rapidly upregulate cell and tissue functions to lay the appropriate foundation for normal ECM reconstruction and tissue regeneration in chronic, nonhealing wounds.

MATERIALS AND METHODS

Recombinant proteins, reagents, and cells

A schematic of the proteins used in this study is shown in Fig. 1A. The recombinant glutathione-S-

Figure 1. Recombinant fibronectin fragments. **(A)** Schematic representation of a fibronectin subunit and recombinant fibronectin fragments. Relevant type III modules are numbered. FNIII1H is the heparin-binding, carboxyl-terminal fragment of FNIII1. **(B)** Aliquots (5 μ g) of purified proteins were electrophoresed on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue. Molecular mass markers (kD) are shown at left. GST, glutathione-S-transferase.

transferase (GST)-tagged fusion proteins FNIII1, FNIII1H, FNIII10, and GST alone were produced in *Escherichia coli*, as described.^{25,26} The RGD chimera, FNIII1H^{RGD} was produced by inserting the integrin-binding RGD loop between the F and G β strands of FNIII1H, analogous to its native location in FNIII10. FNIII1H is a carboxyl-terminal fragment of the first type III repeat of fibronectin and is comprised of amino acids Ile⁵⁹⁷–Thr⁶⁷³ (bases 1802–2032). FNIII1H^{RGD} was produced using the following mutant sense primer: 5'-GGTATACGAGGGCCA GCTCATATCGATTTCAGGGCCGTGGAGACTCGC CGGCAAGCCAAGAAGTGACTCGCTTTGAC-3'. Bases 1976 to 1987 (Q⁶⁵⁵ to H⁶⁵⁸) of FNIII1H were replaced with the underlined bases, which encode for amino acids G¹⁴⁹¹RGDSPAS from FNIII10, leading to a net addition of four amino acids to FNIII1. The FNIII10 insert contains an engineered NgoMIV site as a marker. The BstZ17I site is shown in bold. The antisense primer for FNIII1H^{RGD} (5'-CCC**GAATTC**CTATGTGCTGGT GCTGGTGGTG-3') contains an EcoRI site, shown in bold. The PCR product of this reaction was ligated into pGEX2T/FNIII1H after removal of the corresponding BstZ17I and EcoRI fragment and cloned into DH5 α bacteria.

FNIII1H^{RGD} Δ RRK was produced from pGEX2TK/FNIII1H,8–10 Δ RRK.²⁶ The sense primer 5'-CCC-

GGATCCATCCAGTGAATGCACCACAG-3'

(BamHI site shown in bold) and antisense primer 5'-CCC**GAATTC**CTATGTGCTGGTGGTGGT G-3' (EcoRI site in bold) were used to generate a PCR fragment from this vector comprising bases 1802–2032, and containing the mutations R⁶¹³T, R⁶¹⁵T, K⁶¹⁷A. This fragment was ligated into pGEX2T to generate pGEX2T/FNIII1H Δ RRK. The wild-type BstZ17I-EcoRI fragment from this construct was then replaced with the corresponding fragment from pGEX2T/FNIII1H^{RGD} to generate pGEX2T/FNIII1H^{RGD} Δ RRK. DNA was sequenced to confirm the presence of the mutation. Proteins were isolated on glutathione-Sepharose and dialyzed extensively against phosphate-buffered saline (PBS). Proteins were filter-sterilized and purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie Blue.²² Proteins purity was at least 85% (Fig. 1B) and aliquots were stored at -80°C .

Type I collagen was extracted from rat tail tendons using acetic acid and precipitated with NaCl, as described.²⁸ Fibronectin was purified from outdated human plasma (American Red Cross) using gelatin-Sepharose (GE Healthcare) affinity chromatography.²⁹ Anti-fibronectin polyclonal antibody was from Sigma; anti-GST monoclonal antibody was from Millipore; horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were from BioRad. Hematoxylin and eosin (H&E) were from Leica Microsystems. Unless otherwise noted, chemical reagents were from Sigma.

Mouse embryonic fibronectin-null fibroblasts (FN-null MEFs; obtained from Dr. Jane Sottile, University of Rochester) were cultured at 37°C and 8% CO_2 on type I collagen-coated dishes using a 1:1 mixture of Cellgro[®] (Mediatech) and Aim V (ThermoFisher Scientific). These media do not contain fibronectin and do not require serum-supplementation. Thus, no exogenous source of fibronectin is present during routine culture.²¹ Human mesenchymal stem cells (MSCs) and MSC Growth Medium were from Lonza. MSCs were cultured at 37°C with 5% CO_2 using MSC Growth Media and were used between passage 2 and 5. Tissue culture plates were coated with recombinant fibronectin proteins diluted in PBS at the indicated concentrations for 90 min at 37°C . Unbound protein was removed, wells were washed with PBS, and then blocked with 1% fatty acid-free bovine serum albumin (BSA) for 1 h at 37°C before seeding cells.

Cell adhesion and proliferation assays

Cell adhesion assays were performed as described,²⁶ by seeding FN-null MEFs (1.6×10^5 cells/cm²)

onto tissue culture plates (96-well) precoated with proteins at various concentrations. Cells were incubated for 30 min at 37°C, then washed and fixed with 1% paraformaldehyde. Cell number was quantified by staining with crystal violet. Absorbance at 590 nm was measured to determine relative cell number.

For FN-null MEF growth assays, cells (3.2×10^3 cells/cm²) were seeded onto tissue culture plates (48-well) precoated with fibronectin proteins at indicated concentrations. In some experiments, aliquots of soluble FNIII1H^{RGD}, FNIII10, FNIII1H^{RGD}ΔRRK, or GST (200–800 nM) were added to wells at 20 h postseeding. Cells were incubated for various times at 37°C, 8% CO₂. Cell number was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as described.³⁰ Absorbance at 570 nm was measured and the background absorbance at 700 nm was subtracted from the initial absorbance to determine relative cell number.

For MSC growth assays, MSCs were trypsinized and washed twice with PBS containing soybean trypsin inhibitor. Cells were diluted in serum-free MesencultTM-XF media (Stemcell Technologies) and seeded (3.2×10^3 cells/cm²) onto tissue culture plates (48-well) precoated with test proteins at indicated concentrations. Cells were incubated at 37°C, 5% CO₂ for various lengths of time and cell number was determined using crystal violet staining, as described.²⁶

Solid-phase enzyme-linked immunosorbent assay

Tissue culture plates (96-well) were coated with proteins at indicated concentrations for 90 min at 37°C. Unbound protein was removed and wells were washed with PBS and then blocked with 1% BSA. Bound proteins were detected using an anti-GST monoclonal antibody, followed by HRP-conjugated goat anti-mouse secondary antibody, as described.²⁶ Assays were developed using *o*-phenylenediamine. Absorbance at 450 nm was measured and the absorbance from blank wells was subtracted from initial absorbance to determine the relative amount of protein bound.

Immunoblot analysis

FN-null MEFs were seeded (2×10^4 cells/cm²) on 48-well tissue culture plates precoated with either fibronectin fragments or 50 μg/mL collagen type I. Culture media only was added to “No Cell” control wells. Fibronectin (25 nM) was added to all wells at 4 h postseeding and plates were incubated at 37°C and 8% CO₂ for an additional 20 h. Wells were washed thrice with PBS to remove unbound fibronectin. Aliquots of reduced SDS sample buffer

(0.1 M Tris, 20% glycerol, 4% SDS, 0.2% bromophenol blue, and 4% β-mercaptoethanol) were added to wells and lysates were analyzed by SDS-PAGE and immunoblotting, using enhanced chemiluminescence (ThermoFisher Scientific). Average band intensities for duplicate samples in three independent experiments were determined by densitometry using Carestream Molecular Imaging Software.

Animals

Male BKS.Cg-*Dock7*^{m+/+}*Lepr*^{db}/J mice (32–51 g) (The Jackson Laboratories) between ages 10–16 weeks were used. Mice were housed one per cage; water and laboratory chow were supplied *ad libitum*. Glucose levels were measured weekly using a TRUEtrack[®] Blood Glucose Monitoring System (Nipro Diagnostics). Blood was obtained from the tail vein of anesthetized mice. Mice with glucose levels greater than 300 mg/dL were considered diabetic³¹ and mice exhibiting glucose levels less than 300 mg/dL were excluded from the studies. Mice were weighed daily using an MXX-612 Balance (Denver Instrument) to monitor health status. All experimental animal procedures were reviewed and approved by the University Committee on Animal Resources at the University of Rochester Medical Center.

Three days before surgery, mice were administered acetaminophen (Mapap) at a final concentration of 0.5 mg/mL in water, to achieve a therapeutic dose of ~250 mg/kg, based on water consumption. Mice were anesthetized using a combination of ketamine (120 mg/kg; Hospira) and xylazine (9.6 mg/kg; VEDCO); buprenorphine (0.1 mg/kg) was administered to the mice immediately before surgery. An area on the dorsal flank was shaved and then depilated with Nair[®]. The flank was cleaned with water, followed by iodide and 70% ethanol. Full-thickness skin wounds were made on the dorsal flank using a sterile 6 mm biopsy punch (Miltex). Animals received 1 mL of saline subcutaneously in the scruff of the neck following surgery. Mice continued to receive acetaminophen in their drinking water until day 5 postsurgery.

Recombinant proteins were suspended in PBS to a final concentration of 15 μM. A 15-μL volume of either recombinant protein or PBS alone was pipetted directly onto the wound, and the wounds were immediately covered with Tegaderm (3M Health Care). Mice were anesthetized and proteins or PBS was reapplied on days 2, 4, 7, 9, and 11 after surgery. New Tegaderm was applied following each treatment.

Wound closure

Wound images were obtained immediately following surgery and on treatment days, following removal of the Tegaderm dressing, as described previously.³² Wound closure was measured by tracing the leading edge of epithelium within the wound.^{32,33} The wound area immediately following surgery (day 0) was defined as the original wound area and subsequent wound areas were calculated as a percentage of the original area^{34–36} as follows:

Percent closure = $[1 - (\text{wound area (from leading edge of epithelium)} / \text{original wound area})] \times 100$

Histological analysis

Fourteen days after wounding, animals were sacrificed and 1 cm $\times$ 1 cm sections of skin surrounding the wound were excised down to the fascia, fixed in 10% formalin, and embedded in paraffin. Ten-micrometers sections were cut sequentially through the 1 $\times$ 1 cm skin samples to capture the entire wound space. Four sections were mounted on a slide, and every twentieth slide was stained with H&E to identify the center of the wound. Unstained slides nearest the center of the wound were processed with Masson's Trichrome Stain. Slides were viewed using an inverted microscope (Olympus IX70). Overlapping images of the wound space were obtained with a digital camera (MicroPublisher 3.3; QImaging) and re-constructed using Photoshop (Adobe).

Granulation tissue thickness was measured on day 14 using H&E stained sections obtained from the center of the wound. Granulation tissue thickness was defined as the distance of intact tissue from the bottom of the epidermis to the top of the subcutaneous fat layer and was quantified using ImageJ.

Statistical analysis

Data are presented as mean $\pm$ standard error of the mean and are compiled from at least three separate *in vitro* experiments or five separate animals per treatment condition that underwent surgical procedures on separate days. Statistical comparisons were performed using either a Student's unpaired *t*-test or a one-way analysis of variance followed by the Bonferroni post-test, with GraphPad Prism Version 4 software. Differences were considered significant for *p*-values < 0.05 .

RESULTS

FNIII1H^{RGD} supports cell adhesion, proliferation, and ECM assembly

Previously, a fibronectin fragment was produced in which the RGD loop was inserted into FNIII8,

which was then coupled directly to FNIII1H (FNIII1H,8^{RGD}). As an adhesive substrate, FNIII1H,8^{RGD} supported cell adhesion, proliferation, and ECM assembly.^{26,37} Additionally, soluble FNIII1H,8^{RGD} stimulated granulation tissue deposition in dermal wounds of diabetic mice.³² To further the development of recombinant fibronectin matrix mimetics as supplements to deliver ECM fibronectin signals to chronic wounds, a smaller recombinant chimeric fibronectin fragment, FNIII1H^{RGD} was engineered by removing FNIII8, and instead, inserting the integrin-binding RGD loop directly into FNIII1H between the F and G β strands, analogous to its native location in FNIII10. FNIII1H^{RGD} was developed to provide only the bioactive fragment, FNIII1H, and the canonical RGD attachment sequence to cells, thus minimizing construct size and increasing protein specificity by reducing potential binding sites for other molecules.

Adhesion assays were performed to assess the ability of FNIII1H^{RGD} to support cell adhesion. Results were compared to those obtained with FNIII10, which contains the RGD sequence naturally. FN-null MEFs, which do not produce endogenous fibronectin and are cultured under serum-free conditions, were used for initial adhesion and proliferation studies to analyze responses to FNIII1H^{RGD} in the absence of full-length fibronectin.²¹ No differences were detected in the number of cells adherent to FNIIIH^{RGD} versus FNIII10 at substrate coating concentrations above 25 nM (Fig. 2A), indicating that insertion of RGD sequence into FNIII1H yielded an adhesive protein with properties similar to that of FNIII10. Cells did not adhere to wells coated with saturating concentrations of either FNIII1H or GST (Fig. 2A). An enzyme-linked immunosorbent assay was performed to compare relative ligand densities on tissue culture plates coated with increasing concentrations of either FNIII1H^{RGD} or FNIII10. At coating concentrations greater than 50 nM, no significant differences in protein density were observed (Fig. 2B). Protein density saturated at a concentration of 200 nM for both substrates (Fig. 2B).

Cell number was measured at various times over a 4-day period to assess cell proliferation upon adhesion to FNIII1H^{RGD}. As shown in Fig. 3A, an increase in cell number was observed at each time point after 24 h for cells seeded onto wells pre-coated with saturating concentrations of either FNIII1H^{RGD} or FNIII10. No significant differences in cell number were observed between FNIIIH^{RGD}- and FNIII10-coated wells at any time point (Fig. 3A),

Figure 2. Analysis of cell adhesion and protein adsorption. Tissue culture plates were coated with increasing concentrations (12.5–400 nM) of the various proteins. **(A)** FN-null MEFs were seeded onto protein-coated wells (1.5×10^5 cells/cm²) and allowed to attach for 30 min. The number of adherent cells was determined as described in the Materials and Methods section. In **(B)**, the relative amount of protein bound to wells was determined by enzyme-linked immunosorbent assay. Data are presented as mean absorbance $\pm$ SEM of three individual experiments performed in triplicate. *Significantly different from FNIII10, $p < 0.05$ (ANOVA). ANOVA, analysis of variance; SEM, standard error of the mean.

indicating that FNIII1H^{RGD} supports FN-null MEF proliferation at a rate similar to that of FNIII10. As observed previously (Fig. 2A), cells did not adhere to FNIII1H- or GST-coated wells, and therefore neither substrate supported cell proliferation (Fig. 3A). Addition of soluble FNIII1H^{RGD} to the media of FNIII1H^{RGD}-adherent cells stimulated a further, dose-dependent increase in cell number (Fig. 3B). At the highest concentration tested (800 nM), soluble FNIII1H^{RGD} produced a 1.86 ± 0.09 increase in cell number over control protein addition (Fig. 3B; +FNIII1H^{RGD} vs. +GST). The enhancement of cell proliferation in response to soluble FNIII1H^{RGD} required the inserted RGD loop, as soluble FNIII1H did not increase cell proliferation (Fig. 3C). Importantly, enhanced cell proliferation in response to soluble FNIII1H^{RGD} was mediated by the matricryptic site of FNIII1, as cell proliferation in response to the mutated protein, FNIII1H^{RGD} Δ RRK²² was significantly less than that observed with FNIII1H^{RGD}, and not significantly different from FNIII10 addition (Fig. 3C).

Figure 3. FNIII1H^{RGD} promotes cell proliferation. **(A)** Tissue culture plates were precoated with 400 nM FNIII1H^{RGD}, FNIII10, FNIII1H, or GST. FN-null MEFs were seeded (3.2×10^3 cells/cm²) onto protein-coated wells and incubated for various lengths of time. Cell number was determined using MTT, as described in the Materials and Methods section. Data are presented as mean absorbance of triplicate wells $\pm$ SEM and represent 1 of 3 independent experiments. **(B, C)** Tissue culture plates were precoated with 200 nM FNIII1H^{RGD}. FN-null MEFs were seeded (3.2×10^3 cells/cm²) onto protein-coated wells, allowed to adhere for 20 h, and then treated with **(B)** increasing concentrations of FNIII1H^{RGD} (50–800 nM) or 800 nM GST or **(C)** 200 nM of the various fusion proteins. Cell number was determined 4 days postseeding using MTT. Data are presented as mean absorbance $\pm$ SEM of three **(B)** or five **(C)** experiments performed in triplicate. **(B)** *Significantly different from GST, $p < 0.05$ (ANOVA) **(C)** *Significantly different, $p < 0.05$ (ANOVA); n.s., not significant, $p > 0.05$ (ANOVA). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

The ability of FNIII1H^{RGD} to support cell-mediated ECM remodeling was evaluated next by quantifying the accumulation of exogenously added plasma fibronectin by FN-null cells adherent to FNIII1H^{RGD}. Results were compared to values obtained for cells adherent to either FNIII10 or type I collagen. No significant differences were observed in the amount of ECM fibronectin deposited by cells adherent to FNIII1H^{RGD} versus FNIII10 (Fig. 4). FN-null MEFs adherent to FNIII1H^{RGD}-coated wells deposited significantly less fibronectin than cells adherent to collagen-coated wells (Fig. 4). Minimal fibronectin bound to substrates in the absence of cells (Fig. 4, open bars), demonstrating specificity of fibronectin binding.

To assess the biological activity of FNIII1H^{RGD} in cells capable of fibronectin synthesis, cell proliferation assays were conducted using MSCs. MSCs reside in a variety of tissues in the body and are also present in the wound bed, where they participate in tissue repair.³⁸ FNIII1H^{RGD} supported MSC adhesion and spreading in a dose-dependent manner (Fig. 5A). Four days postseeding, a significant increase in cell number was observed for cells adherent to FNIII1H^{RGD} or FNIII10 versus GST (Fig. 5B). Moreover, by day 7, cell adhesion to FNIII1H^{RGD} resulted in a significant increase in cell number versus adhesion to FNIII10 (Fig. 5B). These data demonstrate that FNIII1H^{RGD} supports MSC proliferation and provides an enhanced

Figure 4. Fibronectin matrix accumulation. FN-null MEFs were seeded (2×10^4 cells/cm²) onto tissue culture plates precoated with FNIII1H^{RGD} (400 nM), FNIII10 (400 nM), or collagen I (50 μ g/mL). Four hours after seeding, fibronectin (12.5 μ g/mL) was added to each well and cells were incubated for an additional 20 h. Fibronectin was also added directly to protein-coated wells to determine binding in the absence of cells ("No Cells"). Lysates were analyzed by immunoblotting using antibodies against fibronectin. Fibronectin band intensities were quantified by densitometry, as described in the Materials and Methods section. Data are presented as mean band intensity normalized to results on the collagen substrate $\pm$ SEM of 3 independent experiments performed in duplicate. *Significantly different, $p < 0.05$ (ANOVA).

proliferation stimulus for MSCs compared with the native FNIII10 module, similar to results shown in Fig. 3C using FN-null MEFs.

FNIII1H^{RGD} accelerates wound closure and increases granulation tissue deposition

Results thus far indicate that FNIII1H^{RGD} supports cell adhesion and ECM deposition, and stimulates cell proliferation. Therefore, we next tested whether topical application of FNIII1H^{RGD} to wounds would accelerate tissue repair in a murine model of chronic wounds.^{33,39} Full-thickness, 6 mm-diameter punch biopsy wounds were made on the dorsal flank of genetically diabetic mice. Wounds were treated topically with soluble FNIII1H, FNIII1H^{RGD}, or GST and wound closure, as denoted by the leading edge of the epithelium, was measured over the course of 2 weeks. Representative images of wounds immediately following surgery ("day 0") and on day 14 after wounding are shown in Fig. 6A. No difference in wound closure rate was observed between wounds treated with the control protein, GST, and the vehicle control, PBS (not shown). Application of FNIII1H^{RGD} to wounds significantly accelerated wound closure compared to GST treatment on days 9 ($73.9\% \pm 4.1\%$ vs. $58.1\% \pm 4.7\%$), 11 ($88.9\% \pm 3.6\%$ vs. $77.1\% \pm 3.3\%$), and 14 ($97.2\% \pm 2.8\%$ vs. $85.2\% \pm 3.5\%$) postwounding (Fig. 6B). Furthermore, wounds treated with FNIII1H^{RGD} exceeded 70% closure 5 days earlier than control (GST)-treated wounds (Fig. 6B; compare FNIII1H^{RGD}, day 9 to GST, day 14). In contrast, application of FNIII1H to wounds did not accelerate wound closure compared to GST (Fig. 6C), even when the treatment dose was increased to 25 μ M (data not shown), indicating that the inserted RGD sequence is necessary for enhanced wound closure.

Granulation tissue deposition at the center of the wound was analyzed 2 weeks after wounding to assess wound healing independent of reepithelialization. Representative H&E-stained tissue sections obtained from the center of day 14 wounds treated with FNIII1H^{RGD} (Fig. 7A) or GST (Fig. 7B) are shown. Wounds treated with FNIII1H^{RGD} contained dense, collagen-rich, granulation tissue that spanned the entire wound space (Fig. 7A). In contrast, GST-treated control wounds contained a thin layer of granulation tissue with limited collagen deposition, particularly toward the center of the wound (Fig. 7B). Granulation tissue deposition was quantified by measuring the thickness of the granulation tissue at the center of the wound, from the epidermis to the subcutaneous fat layer. A significant increase in granulation tissue thickness was observed in response to treatment with FNIII1H^{RGD} compared to

Figure 5. FNIII1H^{RGD} supports MSC adhesion and proliferation. Tissue culture plates were precoated with increasing concentrations of FNIII1H^{RGD} (50–400 nM). MSCs were seeded (1×10^4 cells/cm²) onto protein-coated plates. **(A)** Phase contrast microscopy images were taken at 72 h postseeding. Images represent one of three experiments performed. Scale bar, 50 μ m. **(B)** Cell number was determined at 1, 4, and 7 days postseeding using crystal violet, as described in the Materials and Methods section. Data are presented as mean absorbance $\pm$ SEM of three experiments performed in triplicate. *Significantly different from FNIII1H^{RGD} at corresponding time point, $p < 0.05$ (ANOVA). MSC, mesenchymal stem cell.

Figure 6. FNIII1H^{RGD} accelerates closure of diabetic wounds. Full-thickness, 6 mm punch biopsy wounds on diabetic mice were treated with 15 μ L of 15 μ M FNIII1H^{RGD} ($n=6$), FNIII1H ($n=6$), or GST ($n=6$) immediately after surgery and on days 2, 4, 7, 9, and 11 postsurgery. **(A)** Representative images of wounds immediately following surgery ("day 0") and on day 14. Scale bar = 5 mm. **(B, C)** Percent wound closure was determined. Data are presented as mean value $\pm$ SEM. *Significantly different from GST, $p < 0.05$ (ANOVA).

Figure 7. FNIII1H^{RGD} increases granulation tissue deposition. Full-thickness punch biopsy wounds on diabetic mice were treated with 15 μ M FNIII1H^{RGD} (**A**; $n=6$) or GST (**B**; $n=9$), as described in the legend to Fig. 6. Fourteen days after surgery, animals were sacrificed and the skin surrounding the wound was excised and sections were processed for hematoxylin and eosin. (**A**, **B**) Representative sections obtained from the center of the wound. Scale bar = 500 μ m. (**C**) Granulation tissue thickness was quantified at the center of the wound for each mouse as described in the Materials and Methods section. Data are presented as mean granulation tissue thickness $\pm$ SEM. *Significantly different from GST, $p < 0.05$ (ANOVA). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

GST controls ($508.2 \pm 131.7 \mu\text{m}$ vs. $176.8 \pm 33.0 \mu\text{m}$, respectively) (Fig. 7C). As a comparison, we previously reported average granulation tissue thickness of day 11, fully closed wounds from wild-type mice to be $775.2 \pm 71.75 \mu\text{m}$,³² indicating that FNIII1H^{RGD} did not stimulate a hypertrophic response. Finally, granulation tissue of wounds treated with FNIII1H^{RGD} showed functional, erythrocyte-containing blood vessels at both the periphery and center of the wound space (Fig. 8, arrows), indicating that the granulation tissue formed in response to FNIII1H^{RGD} treatment supported revascularization.

DISCUSSION

In this study, we describe the functional characterization of a novel, chimeric fibronectin matrix

analog and show that application of soluble FNIII1H^{RGD} to full-thickness excisional wounds in diabetic mice accelerates wound closure and promotes granulation tissue formation. The ability of FNIII1H^{RGD} to enhance dermal repair was assessed in a well-established model of impaired healing that exhibits many of the clinical findings seen in diabetic patients.^{33,40} Direct application of FNIII1H^{RGD} to diabetic wounds significantly increased the extent of wound closure by day 9 compared to controls, and by day 14, the epithelium was multilayered. Moreover, treatment with FNIII1H^{RGD} significantly increased granulation tissue deposition over control-treated diabetic wounds. The granulation tissue of wounds treated with FNIII1H^{RGD} was completely covered by epithelium, and characterized by dense collagen,

Figure 8. Revascularization of diabetic wounds treated with FNIII1H^{RGD}. Full-thickness punch biopsy wounds on diabetic mice were treated with 15 μ M FNIII1H^{RGD} (**A**; $n=6$) or GST (**B**; $n=6$), as described in the legend to Fig. 6. Fourteen days after surgery, animals were sacrificed and the skin surrounding the wound was excised and processed for histological analysis. Ten micrometers sections corresponding to the center of the wound were stained using Masson's Trichrome. Representative images shown were obtained from peripheral and central fields of view of the wound. Arrows indicate blood vessels. Scale bar = 50 μ m. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

increased cellularity, and the presence of functional blood vessels. These studies provide evidence that FNIII1H^{RGD}, designed to support cell adhesion via the RGD loop while delivering ECM fibronectin-specific signals to cells via the bioactive matricryptic site in FNIII1, can promote the healing of chronic diabetic wounds.

Therapeutic protein engineering is an emerging field that combines genetic, biochemical, and functional information to improve existing proteins or invent new protein technologies. Using these principles, we developed an approach to deliver ECM fibronectin-specific signals to cells in a soluble form. By identifying the functional aspects of ECM fibronectin essential for wound repair and then distilling those components into a single recombinant molecule, biologically active, small molecule analogs of ECM fibronectin were created. In earlier studies, we localized some of the ECM-specific effects of fibronectin to a cryptic heparin-binding site in FNIII1.^{19,22,25} This conformation-dependent site is not exposed in soluble fibronectin,⁴¹ but it becomes unmasked either during fibronectin matrix polymerization or as cells exert tension on insoluble fibronectin fibrils.⁴² To mimic fibrillar fibronectin having an "open" conformation of FNIII1, the first two β strands of FNIII1 were deleted, leaving the heparin-binding sequence, Arg-Trp-Arg-Pro-Lys (RWRPK) exposed. The fibronectin matrix mimetic proteins bind to cell surface heparan sulfate proteoglycans and integrins^{19,22,25} and stimulate cell growth, migration, and contractility to a similar or greater extent than full-length ECM fibronectin.²⁶ Thus, our protein engineering approach effectively bypasses the requirements

for soluble fibronectin to undergo cell-mediated conformational changes and assembly into insoluble fibrils to become biologically active.

After tissue injury, loss of the preexisting connective tissue matrix, coupled with an inability of cells to reconstruct a fibrillar fibronectin and collagen matrix with adequate tensile strength would be expected to perpetuate tissue loss through further decreases in cell adhesion, contractility, proliferation, and matrix deposition. Moreover, decreasing intracellular cytoskeletal tension preferentially reduces the appearance of an FNIII1 epitope in ECM fibronectin.⁴² Thus, reduced cellular tension on ECM fibrils within chronic wounds may also impair tissue regeneration by "closing" matricryptic FNIII1 sites that would normally stimulate cell growth, collagen fibril contraction, and reepithelialization of injured tissues. Providing injured cells with engineered fibronectin matrix mimetics containing "open" FNIII1 sites may circumvent either diminished fibronectin matrix assembly or decreased expression of matricryptic FNIII1 sites and hence, accelerate wound closure and granulation tissue deposition by providing tissues with ECM fibronectin-specific signals and structural cues.

Small, soluble, recombinant analogs of ECM fibronectin provide a new approach to deliver specific signals from insoluble fibronectin fibrils to chronic wounds. Results in this study are similar in magnitude to effects of equimolar platelet-derived growth factor (PDGF) on granulation tissue formation of full-thickness wounds in diabetic mice.³³ In addition, application of FNIII1H^{RGD} significantly increased wound closure by day 9, whereas

significant differences in wound closure in response to PDGF were not observed until 21 days after injury.³³ Recombinant fibronectin fragments have also been developed to enhance growth factor delivery and/or activity to cells.^{43,44} Treatment of diabetic mouse wounds with a fragment of fibronectin that couples the integrin-binding domain (FNIII9–10) with the C-terminal growth factor-binding domain (FNIII12–14) enhanced the healing effects of combined PDGF and vascular endothelial growth factor (VEGF) treatment.⁴⁵ Of note, granulation tissue deposition in response to treatment with both FNIII9–10/12–13 and PDGF/VEGF was similar to that observed in this study (Fig. 7), where FNIII1H^{RGD} was added in the absence of additional growth factors. Thus, FNIII1H^{RGD} holds promise as a new category of supplemental treatment approaches for chronic diabetic wounds.

INNOVATION

Nonhealing and hard-to-heal wounds are becoming increasingly prevalent and effective treatment options are limited. FNIII1H^{RGD} mimics the insoluble, fibrillar form of a naturally occurring ECM protein involved in wound repair. FNIII1H^{RGD} is small (~11 kDa), providing increased structure, stability, and activity over short peptides. FNIII1H^{RGD} is easily produced using common recombinant protein techniques, and it could be integrated into a variety of delivery vehicles and biomaterials to promote healing of chronic wounds. If successful, recombinant, soluble fibronectin matrix mimetics will help to fill a critical need for new and innovative approaches to wound repair.

KEY FINDINGS

- A novel chimeric fibronectin protein was produced by inserting the adhesive RGD loop into the bioactive FNIII1H fragment.
- FNIII1H^{RGD} supports cell adhesion, proliferation, and ECM fibronectin deposition.
- Application of soluble FNIII1H^{RGD} to full-thickness cutaneous wounds in diabetic mice accelerates wound closure and stimulates granulation tissue deposition.

ACKNOWLEDGMENTS AND FUNDING SOURCES

This study was supported in part by NIH grant R01 EB018210 and by a Technology Development Grant from the University of Rochester. The authors thank Susan Wilke-Mounts for excellent technical assistance.

AUTHOR DISCLOSURE AND GHOSTWRITING

No competing financial interests exist. The authors listed wrote the content of this article. No ghostwriters were used to write this article.

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Abbreviations and Acronyms

BSA = bovine serum albumin
 ECM = extracellular matrix
 FNIII = fibronectin type III repeat
 GST = glutathione-S-transferase
 MSC = mesenchymal stem cell
 MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
 PAGE = polyacrylamide gel electrophoresis
 PBS = phosphate-buffered saline
 PDGF = platelet-derived growth factor
 RGD = Arg-Gly-Asp
 SDS = sodium dodecyl sulfate
 VEGF = vascular endothelial growth factor