A modified collagen gel enhances healing outcome in a preclinical swine model of excisional wounds

Haytham Elgharably, MD; Sashwati Roy, PhD; Savita Khanna, PhD; Motaz Abas, BS; Piya DasGhatak, MS; Amitava Das, MS; Kareem Mohammed, MD; Chandan K. Sen, PhD

Departments of Surgery, Davis Heart & Lung Research Institute, Center for Regenerative Medicine and Cell-B Therapies and Comprehensive Wound Center, The Ohio State University Wexner Medical Center, Columbus, Ohio

Reprint requests: Prof. C. K. Sen, 473 West 12th Avenue, 512 DHLRI, The Ohio State University Medical Center, Columbus, OH 43210. Tel: +1 614 247 7658; Fax: +1 614 247 7618; Email: chandan.sen@osumc.edu

Manuscript received: March 7, 2012
Accepted in final form: January 31, 2013

DOI:10.1111/wrr.12039

ABSTRACT

Collagen-based dressings are of great interest in wound care. However, evidence supporting their mechanism of action is scanty. This work provides first results from a preclinical swine model of excisional wounds, elucidating the mechanism of action of a modified collagen gel (MCG) dressing. Following wounding, wound-edge tissue was collected at specific time intervals (3, 7, 14, and 21 days post-wounding). On day 7, histological analysis showed significant increase in the length of rete ridges, suggesting improved biomechanical properties of the healing wound tissue. Rapid and transient mounting of inflammation is necessary for efficient healing. MCG significantly accelerated neutrophil and macrophage recruitment to the wound site on day 3 and day 7 with successful resolution of inflammation on day 21. MCG-induced monocyte chemotactic protein-1 expression in neutrophil-like human promyelocytic leukemia-60 cells in vitro. In vivo, MCG-treated wound tissue displayed elevated vascular endothelial growth factor expression. Consistently, MCG-treated wounds displayed significantly higher abundance of endothelial cells with increased blood flow to the wound area indicating improved vascularization. This observation was explained by the finding that MCG enhanced proliferation of wound-site endothelial cells. In MCG-treated wound tissue, Masson’s trichrome and picrosirius red staining showed higher abundance of collagen and increased collagen type I:III ratio. This work presents first evidence from a preclinical setting explaining how a collagen-based dressing may improve wound closure by targeting multiple key mechanisms. The current findings warrant additional studies to determine whether the responses to the MCG are different from other collagen-based products used in clinical setting.

Chronic ulcers represent a major clinical challenge to wound care providers. Patients suffering from nonhealing ulcers are prone to serious complications such as secondary infection and amputation, which are associated with high rates of mortality. Chronic wounds pose a substantial burden on the health system in the United States, with $5–10 billion being spent annually for management of these wounds. The development of effective and clinically relevant therapeutic interventions thus represents a matter of high priority.

The extracellular matrix (ECM) is a key compartment in the healing tissue. In addition to providing structural support to the healing tissue, the ECM elicits cell signaling aimed at executing the healing response.1 Fibrillar collagen is the most abundant, extracellular ECM of the skin, representing 25–35% of the whole-body protein content. The three-dimensional collagen architecture defines mechanical tissue properties, i.e., stiffness and porosity, which guide or oppose cell migration and positioning in different contexts, such as regeneration and immune response. Because of such properties, collagen-based dressings have been of interest in wound care.2 In addition to being an inducer of cell signaling, collagen-based dressings may improve healing outcomes by deactivating excessive matrix metalloproteases by acting as a sacrificial substrate.3 Collagen dressings may also recruit several cell types to the wound site, facilitating granulation tissue formation. Furthermore, such dressings may help maintain moist wound environment by absorbing wound exudates.4 Several efforts are currently in progress to optimize the composition and formulation of collagen dressings.5 In this work, we elucidate the mechanism of action of a gel formulated to produce a highly concentrated dispersion of modified collagen.

MATERIALS AND METHODS

Swine excisional wound model and treatment

Domestic white pigs were used in this study. All experiments were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee. Pigs (70–80 lb) were sedated by Telazol (Fort Dodge Animal Health, Fort Dodge, IA) and anesthetized by mask with isoflurane (3–4%). The dorsal region was shaved. The skin was surgically prepared with alternating chlorhexidine 2% and alcohol.
70% (Butler Schein, Columbus, OH) scrubs. Under such aseptic conditions, four sets of full-thickness excisional wounds were established on the back of pigs using a 3-mm disposable biopsy punch. The depth of the wound is measured by the length of stainless steel section of the punch biopsy (7 mm). The wounds were created by cutting through the skin until the entire length of the stainless steel section is below the skin and the plastic shoulders (edges) are at the surface of the skin, with that length being enough to reach the subcutaneous fat in all wounds. A total of four sets with eight wounds in each set were created on each side of the back. A scaled plastic template was used to create the wounds at fixed distance from each other and from the spine (+cm), starting below the lower border of the scapula on the dorsum of the pig. Wounds from one side of the back were treated with a modified collagen gel (MCG) followed by dressing with Tegaderm (3M, St. Paul, MN), while the wounds from the contralateral side of the back were dressed with Tegaderm™ only and received no other treatment (control). In all pigs, control and treated wounds were created on different sides in the same animal, with all applications to the wounds maintained constant, ensuring exposure to similar biological effects. Treatment sides were alternated among the animals to avoid any side-specific effect. Wounds of the treatment side were retreated with MCG at 24 and 72 hour postwounding. On specified time points (days 3, 7, 14, and 21 postwounding), the entire wound tissue was harvested using a 6-mm disposable biopsy punch for subsequent analyses. Animals were maintained on 12-hour light–dark cycles and were euthanized after the completion of experiments. MCG was provided as Stimulen™ gel by Southwest Technologies Inc. (North Kansas City, MO). According to the manufacturer, the unique formulation of the MCG represents a mixture of 52% collagen of long and short polypeptides along with glicerine, water, and fragrance. The MCG is a highly concentrated modified collagen (mainly type I) in a gel form. We performed a proteomics analysis to determine the components present in the MCG.

Proteomic analysis

Sample preparation and mass spectrometry (MS) analysis

Proteins/peptides in MCG were purified using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After tryptic digestion of the purified proteins, the MS/MS spectra were obtained using capillary-liquid chromatography tandem mass spectrometry (MS/MS). A Thermo Finnigan linear trap quadrupole (LTQ) mass spectrometer equipped with a CaptiveSpray source (Bruker-Michrom, Billerica, MA) in positive ion mode was used. The LC system was an UltiMate 3000 system from Dionex (Sunnyvale, CA). The solvent A was water containing 50 mM acetic acid and the solvent B was acetonitrile. Five microliter of each sample was first injected onto the μ-Precolumn Cartridge (Dionex) and washed with 50 mM acetic acid. The injector port was switched to inject and the peptides were eluted off of the trap onto the column. A 0.2 × 150 mm, 3u, 200 A, Magic C18 (Bruker-Michrom) was used for chromatographic separations. Peptides were eluted directly off the column into the LTQ system using a gradient of 2–80% solvent B over 45 minutes, with a flow rate of 2 μL/min. The total run time was 65 minutes. The MS/MS was acquired according to standard conditions established in the laboratory. Briefly, a Capitve-Spray source operated with a spray voltage of 3 kV and a capillary temperature of 200 °C is used. The analysis was programmed for a full scan recorded between 350 and 2,000 Da and a MS/MS scan to generate product ion spectra to determine amino acid sequence in consecutive instrument scans of the 10 most abundant peaks in the spectrum.

Data processing and analysis

Sequence information from the MS/MS data was processed by converting the .raw files into a merged file (.mgf) using an in-house program. The resulting .mgf files were searched using Mascot Daemon by Matrix Science version 2.3.2 (Boston, MA) and the database searched against the full Swiss-Prot database version 2012_06 (536,489 sequences; 190,389,898 residues) or National Center for Biotechnology Information database version 20120515 (18,099,548 sequences; 6,208,559,787 residues). The mass accuracy of the precursor ions was set to 1.8 Da and the fragment mass accuracy was set to 0.8 Da. Considered variable modifications were methionine oxidation and deamidation. Fixed modification for carbamidomethyl cysteine was considered. Two missed cleavages for the enzyme were permitted. A decay database was searched to determine the false discovery rate (FDR), peptides were filtered according to the FDR, and proteins identified required bold red peptides. Protein identifications were checked manually and proteins with a Mascot score of 50 or higher with a minimum of two unique peptides from one protein having a -b or -y ion sequence tag of five residues or better were accepted and have been presented in Table 1.

Histology

Formalin-fixed paraffin-embedded or optimal cutting temperature–embedded frozen wound-edge specimens were sectioned. The paraffin sections were deparaffinized and stained with hematoxylin and eosin, Masson’s trichrome, or picrosirius red staining (PRS) using standard procedures. Immunohistochemical staining of paraffin or frozen sections was performed using the following primary antibodies: keratin 14 (1:600; K14; Thermo Fisher Scientific Inc., Waltham, MA), 4’,6-diamidino-2-phenylindole (DAPI) (1:10,000; Life Technologies, Grand Island, NY), antityrosinoperoxidase (anti-MPO) (1:400; DAKO North America Inc., Carpinteria, CA), antimacrophage L1 calprotectin (1:400; MAC387; Thermo Fisher Scientific Inc.), anti-von Willebrand’s Factor (vWF) (DAKO North America Inc.), and anti-Ki67 (1:400, Thermo Fisher Scientific Inc.) after heat-induced epitope retrieval when necessary. Secondary antibody detection and counterstaining were performed as described previously.

Image quantification

Mosaic images of whole wounds were collected under 20x magnification guided by MosaiX software (Zeiss, Thorn-
wood, NY) and a motorized stage. In order to cover the whole wound, each mosaic image was generated by combining a minimum of ~100 images. Between seven and nine high-powered representative areas from mosaic images were quantified for each time point. Image analysis was performed by employing Automatic software (Zeiss) for quantification of the percentage of immune-histochemical reaction positive areas (expressed as % area).

Laser speckle contrast imaging (LASCI) technology
PeriCam PSI System is a blood perfusion imager based on laser speckle technology. The system allows for visualization and quantification of microcirculation in tissues on a real-time basis. 10 The mean blood flow was measured in excisional wounds within a 100-mm² surface area (resolution 0.54 x 0.54 mm, working distance 1720 cm, 55 images/second).

Cell culture, differentiation, and treatment
The human promyelocytic leukemia cells (HL)-60 (American Type Culture Collection [ATCC], Manassas, VA; ATCC code CCL-240) were cultured in RPMI 1640 with L-glutamine, supplemented with 20% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) ( Gibco, Auckland, New Zealand). For differentiation of HL-60 cells into mature neutrophils, cells were first suspended in RPMI 1640 media, supplemented with 1% PS and 20% heat-inactivated FBS ( Gibco), followed by addition of 10 μM 13-cis-retinoic acid (Thermo Fisher, Bridgewater, NJ). Cultures were incubated at 37°C for 5 days. Culture media were changed every third day and fresh retinoic acid was added. Human acute monocytic leukemia cell line (THP)-1 monocytes (ATCC) were cultured and differentiated to macrophages as described previously. 11 A solution of MCG stock was prepared by dissolving 1 g of MCG per milliliter of culture media. To treat differentiated cells with MCG, 100 μL of MCG stock was added to culture plates containing 900 μL media.

RNA isolation
Immediately after collection, wound tissue biopsies were rinsed in saline, patted dry, and snap frozen in liquid nitrogen. Grinding of tissues was performed using 6770 Freezer/Mill® cryogenic grinder (SPEX SamplePrep, Metuchen, NJ). Total RNA from tissue or cultured cells were extracted using mirVana RNA isolation kit (Ambion, Austin, TX) as described. 11, 12

Reverse transcription and quantitative real-time polymerase chain reaction (PCR)
Tissue messenger RNA was quantified by real-time or quantitative PCR assay using the double-stranded DNA binding dye SYBR Green-I as described previously. 12 The primer set used for the individual genes is listed below. 18s ribosomal RNA was used as a reference housekeeping gene.

Human_VEGF-A165 5′-TGC CCA CTG AGG AGT CCA ACA T-3′
Human_VEGF-A165 5′-CAC GTC TGC GGA TCT TGT ACA AAC A-3′
Pig_VEGF-A 5′-CTC TCT CCT ACT TGT ACT GGT CCT T-3′
Pig_VEGF-A R 5′-TTA TTT CAA AGG AAT GTG TGG CG-3′
Pig_vWFF 5′-GGC TCT GAT AAG CTG TCC GAG G-3′
Pig_vWFR 5′-TTT CGG TCC TGG AGC GAG A-3′
Human_COL1A 5′-ACG TCC TGG TGA AGT TGG TC-3′

Table 1. Proteomic analysis of MCG components

<table>
<thead>
<tr>
<th>Description</th>
<th>Accession</th>
<th>Unigene ID</th>
<th>Mass (Da)</th>
<th>Number of significant sequences</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin subunit beta</td>
<td>HBB_BOVIN</td>
<td>Bt.23726</td>
<td>16,001</td>
<td>7</td>
<td>685</td>
</tr>
<tr>
<td>Carbonic anhydrase 2</td>
<td>CAH2_BOVIN</td>
<td>Bt.49731</td>
<td>29,096</td>
<td>10</td>
<td>650</td>
</tr>
<tr>
<td>Collagen alpha-1 (I) chain</td>
<td>CO1A1_BOVIN</td>
<td>Bt.23316</td>
<td>139,880</td>
<td>3</td>
<td>321</td>
</tr>
<tr>
<td>Hemoglobin subunit alpha</td>
<td>HBA_BOVIN</td>
<td>Bt.10591</td>
<td>15,175</td>
<td>5</td>
<td>319</td>
</tr>
<tr>
<td>Peroxiredoxin-2</td>
<td>PROX2_BOVIN</td>
<td>Bt.2689</td>
<td>22,217</td>
<td>5</td>
<td>308</td>
</tr>
<tr>
<td>Alpha-1-antiproteinase</td>
<td>A1AT_BOVIN</td>
<td>Bt.982</td>
<td>40,417</td>
<td>2</td>
<td>220</td>
</tr>
<tr>
<td>Serpin A3-7</td>
<td>SPA37_BOVIN</td>
<td>Bt.55387</td>
<td>47,140</td>
<td>3</td>
<td>161</td>
</tr>
<tr>
<td>Collagen alpha-1 (III) chain</td>
<td>CO3A1_BOVIN</td>
<td>Bt.64714</td>
<td>93,708</td>
<td>2</td>
<td>147</td>
</tr>
<tr>
<td>Collagen alpha-2 (I) chain</td>
<td>CO1A2_BOVIN</td>
<td>Bt.53485</td>
<td>129,499</td>
<td>2</td>
<td>103</td>
</tr>
<tr>
<td>Serpin A3-3</td>
<td>SPA33_BOVIN</td>
<td>Bt.55387</td>
<td>46,411</td>
<td>2</td>
<td>85</td>
</tr>
<tr>
<td>Actin, aortic smooth muscle</td>
<td>ACTA_BOVIN</td>
<td>Bt.37349</td>
<td>42,381</td>
<td>2</td>
<td>79</td>
</tr>
</tbody>
</table>

*Most abundant proteins as detected using proteomics analysis are presented. Two unique peptides from one protein having a b or y ion sequence tag of five residues or better were accepted.
MCG, modified collagen gel.
The wound neutrophil count on day 3 postwounding was performed using antibody against MPO, a lysosomal enzyme stored in azurophilic granules of the neutrophil (Figure 2A).13 Quantitation of MPO positive areas in mosaic images of the wound-edge tissue sections showed higher abundance of neutrophils in wounds treated with MCG (Figure 2B). To determine the effect of MCG on neutrophil function, we treated HL-60-derived neutrophils with MCG. Such treatment significantly enhanced the release of MCP-1 by these cells (Figure 2C). This observation led to the hypothesis that MCG would be effective in attracting more macrophages to the wound site via increased MCP-1 release. Next, macrophages were identified in wound tissue sections using antibody against the macrophage L1 protein/calprotectin (Figure 3A), which has been reported to be a reliable marker for macrophages in swine tissue.14 The kinetics of macrophage infiltration to the excisional wound site was evaluated over the duration of the healing process. Compared with untreated wounds, MCG-treated wounds showed significant increase in macrophage infiltration at day 3 postwounding. Higher macrophage abundance was observed in wounds treated with MCG compared to untreated wounds. (Figure 3B, C).

**Enzyme-linked immunosorbent assay (ELISA)**

Monocyte chemotactic protein-1 (MCP-1) level in culture media was determined using commercially available ELISA kit as per manufacturer's recommendation (R&D Systems, Minneapolis, MN).

**Statistical analyses**

Data are reported as mean ± standard deviation of at least three to four animals as indicated. Because the data were not normally distributed, non-parametric statistics was used. The significance of differences between control and treated groups was evaluated using the two-tailed Mann–Whitney test; p < 0.05 was considered statistically significant.

**RESULTS**

We chose a preclinical model of full-thickness dermal excisional wounds to study the effect of a MCG on wound healing outcomes. Proteomics analysis of MCG shows that collagen (I and III), hemoglobin, and peroxiredoxin are major components present in this gel formulation (Table 1). This study was designed to characterize specific phases of the wound healing process by harvesting wound tissue at several time points during the course of the healing. Specifically, we collected the wound-edge tissue biopsies as shown in Figure 2A and B. Histological characterization of wound reepithelialization was performed by staining the wound tissue for keratin 14, the type I cytokeratin that forms the cytoskeleton of epithelial cells (Figure 1). Tissue sections were counterstained with DAPI to visualize nuclei. Lengths of the rete ridges were measured using an automated unbiased approach by utilizing an Automeasure software (Zeiss). MCG-treated wounds displayed longer rete ridge structures compared with untreated wounds.

Figure 1. Wound reepithelialization. Representative images showing increased length of rete ridges in the MCG-treated excisional wounds on day 7 postwounding. OCT embedded frozen wound biopsies were sectioned (8 µm) and stained using anti-keratin-14 (K14, green) and DAPI (blue) immunofluorescence staining. The arrows indicate the two edges of the wound. Bar graph presents the length of rete ridges within wound as quantified using Mosaic software (Zeiss). Scale bar, 500 µm. Data are mean ± SD (n = 4); *p < 0.05 compared to untreated control wounds.

Figure 2. Increased neutrophil infiltration in excisional wounds treated with modified collagen gel (MCG). (A) Formalin-fixed paraffin-embedded (FFPE) wound biopsies were sectioned (6 µm) and stained using anti-myeloperoxidase (MPO, dark brown). The sections were counterstained with hematoxylin (blue). Scale bar, 1 mm. W, wound; TD, Tegaderm; MCG, modified collagen gel. Right panels are the zoom of the boxed area of images shown in left panels. Scale bar, 100 µm. (B) Bar graph shows quantitation of neutrophils at day 3 postwounding in MCG-treated or -untreated control excisional wounds. Data are presented as mean ± SD (n = 4); *p < 0.05 compared to untreated wounds. (C) Release of monocyte chemotactic protein-1 (MCP-1) from HL60-derived human neutrophils treated with modified collagen gel (MCG). HL60 cells were differentiated into neutrophils and incubated for 24h with or without MCG. MCP-1 concentration in culture media was measured using enzyme-linked immunosorbent assay. Data are mean ± SD (n = 4); *p < 0.05 compared to untreated cells.
age abundance at the MCG-treated wound site was also noted in day 7 postwounding. However, on day 21 postwounding, the macrophage count of MCG-treated wounds reduced markedly and were comparable with that of control wounds, indicating effective resolution of inflammation (Figure 3B).

Interestingly, in studies using cultured macrophages, MCG was found to potently induce vascular endothelial growth factor (VEGF) gene expression (Figure 3C). VEGF is a known potent angiogenic factor that is secreted into the wound tissues to promote tissue vascularization. On day 7 postwounding, VEGF gene expression in the wound-edge tissue was noted to be significantly higher for MCG-treated wounds (Figure 4). Consistently, abundance of endothelial cell-specific gene vWF was higher in the wound-edge tissue on day 21 postwounding. VWF is a glycoprotein that is produced by endothelial cells and is routinely used to identify vascular structures in tissue sections (Figure 4).

During the proliferative phase of wound healing, angiogenesis is necessary to supply oxygen, nutrients, and other blood-borne factors required for cell migration and proliferation. Wound tissue vascularization was evaluated through quantitative measurement of endothelial cell abundance and blood flow in pair-matched wounds. Quantitative analyses of vWF-positive areas in wound sections showed significant increase of endothelial cell abundance in day 21 postwounding tissue collected from MCG-treated wounds (Figure 5A). Interestingly, MCG-treated wounds displayed more mature vascular formations of vWF-positive cellular structures compared with untreated wounds. Functional assessment of wound tissue vascularization was accomplished by imaging blood flow using laser speckle technology in vivo. MCG-treatment was observed to significantly increase wound-site blood flow compared with corresponding pair-matched control wound tissue (Figure 5B). The striking effect of MCG on wound angiogenesis led us to evaluate underlying mechanisms. Increased endothelial cell proliferation and migration are two key events that lead to enhanced angiogenesis. Thus, we studied wound-site endothelial cell proliferation using Ki67 and vWF double immune staining, followed by automated quantification of colocalized signals in thomosis images. Ki67 is a nuclear protein that is associated with cellular proliferation. Marked increase of the proliferating endothelial cells was observed in MCG-treated wounds (Figure 6).

An important aspect of the proliferative phase of wound healing is collagen deposition, which is responsible for
Figure 5. Increased vascularization in excisional wounds treated with modified collagen gel (MCG). (A) Representative immunofluorescence images from wound sections (8 μm) stained using von Willebrand’s Factor (vWF; red) and DAPI (blue). Inset shows the lumen like structure counted for the adjacent quantitative graph presented. Scale bar, 50 μm. Bar graphs (top and middle) show quantitation of the lumen like structures in each group (top bar graph) and endothelial cell in MCG-treated or untreated excisional wounds day 21 postwounding (middle bar graph). Data are presented as mean ± SD (n = 3); *P < 0.05 compared with untreated wounds. (B) Laser speckle images of MCG-treated or control excisional wounds on day 21 postwounding. Left panels are speckle flow color images (yellowish-green color represents increased blood flow), while right panels are black/white contrast images (red dashed circles indicate the site of excisional wounds). Scale bar, 2.4 mm. Bar graph (bottom) represents the quantitative data from laser speckle analysis. Data presented as mean ± SD (n = 4); *P < 0.05 compared with untreated wounds. DAPI, 4′,6-diamidino-2-phenylindole; SD, standard deviation; W, wound area; TD, Tegaderm.

Figure 6. Increased proliferation of wound endothelial cells in modified collagen gel (MCG) treated wounds. Representative immunofluorescence images of wound sections (8 μm) stained using Ki67 (marker of proliferating cells, green) and von Willebrand’s Factor (vWF) (endothelial cells, red). Compared with untreated excisional wounds, the MCG-treated wounds showed increased number of vWF-positive proliferating cells (yellow areas in merged images). The bottom panels are zoom of dashed white box in middle panels. Top and middle panels: scale bars, 100 μm. Bottom panel: scale bar, 20 μm. TD, Tegaderm.

tensive strength of the postheal tissue. Greater tensile strength of the postheal tissue ensures effective protection against reopening of a close wound in response to shear stress. Quantitative and qualitative analyses of collagen formation in MCG-treated or untreated wound were done using wound-edge tissue harvested on day 21 postwounding. Initial histological examination of wound-edge tissue using Masson’s trichrome staining displayed higher abundance of mature collagen fibers in MCG-treated wounds (Figure 7A and C). PRS was used to distinguish between type I and type III collagen in the wound-edge tissue. After staining with PRS, collagen type I (thick fibers) displays yellow-orange birefringence, while type III (thin fibers) displays green birefringence when viewed under polarized light microscope. Collagen type I:III ratio was significantly increased in MCG-treated wounds compared with untreated wounds (Figure 7B and D). These histological findings were supported by measurement of collagen type I gene expre-
Figure 7. Increased collagen abundance in excisional wounds treated with modified collagen gel (MCG). (A) Representative images of formalin-fixed paraffin-embedded (FFPE) wound biopsy sections (5 μm) stained using Masson’s trichrome staining. This staining results in blue–black nuclei, blue collagen, and light red or pink cytoplasm cyttoplasm. Epidermal cells appear reddish. Scale bar, 500 μm. The arrows indicate the edges of the wound. Right panels are the zoom of boxed area of images shown in left panel. Scale bar, 50 μm. (B) Representative images from FFPE wound tissue biopsy sections stained using picrosirius red staining (PFRS). This stain can be used to distinguish between type I and type III collagen in wound tissues; type I (thick fibers) appears yellow–orange birefringence, while type III (thin fibrils) appears green birefringence when viewed under polarized light microscope. (C) Bar graph shows quantitation of collagen abundance in MCG-treated or untreated control wounds on day 21 postwounding. Data are presented as mean ± SD (n = 3); *p < 0.05 compared with untreated wounds. (D) Bar graph shows quantitation of the ratio of collagen types I and III. Scale bar, 75 μm. Data are mean ± SD (n = 3); p < 0.05 compared with untreated control wounds. (E) Collagen type I gene expression in excisional wounds treated with MCG. The collagen type I gene expression in day 14 wound tissues was quantified using real-time PCR. Gene expression data are presented as percentage change compared with MCG-untreated control wound tissues. Data are mean ± SD (n = 4); p < 0.05, SD, standard deviation; W, wound; PCR, polymerase chain reaction; rRNA, ribosomal RNA; TD, Tegaderm.

stockpiled for emergent needs. On scientific considerations, although observational studies report favorable outcomes, the underlying mechanisms of action remain speculative and not established in wound healing studies. This work is the first to address such mechanisms in a preclinical swine model, which is known to most closely resemble the human wound. Anatomically as well as physiologically, pig skin closely resembles human skin. Additionally, pigs and humans have similar physical and molecular responses to various growth factors. Thereby, the swine model is generally accepted as an excellent tool to study wound healing.

Rete ridges are epidermal protrusions that extend downward between dermal papillae into the connective tissue of the skin. Rete ridges interlock the dermal-epidermal junction. Epidermal cells receive nutrients from the blood vessels in the dermis. Rete ridges increase the surface area of the epidermis that is exposed to these blood vessels and blood-borne nutrients. In addition, effective interlocking of the dermal-epidermal junction by rete ridges protects the skin from reopening in response to shear stress. With aging, the rete ridges are known to shorten, making the skin fragile. Shortening of the rete ridges limits nutrient supply to the epidermis by decreasing the surface area in contact with the dermis, also interfering with the skin’s normal repair process. Our observation that a collagen dressing may increase the size of rete ridges in the healing wound tissue provides first evidence demonstrating improved quality of closure, reestablishing more healthy skin featuring well-nourished epidermis, and that is potentially more resistant to reopening of the wound in response to shear stress.

Collagen may serve as neutrophil chemotactic factor. Neutrophils actively migrate actively into three-dimensional gels of collagen. Studies on lung injury have showed that degraded collagen may support neutrophil chemotaxis. Consistently, we provide first evidence demonstrating that a collagen-based dressing may improve neutrophil chemotaxis into the wound site. Neutrophils are known to be functionally activated when in contact with collagen I, the primary ingredient in MCG. It is therefore plausible that MCG will help the wound tissue fight infection through bolstered neutrophil function. MCP-1 or chemokine (C-C motif) ligand 2 is a major driver of wound inflammation. Neutrophil derived MCP-1 play a key role in recruiting macrophages to the wound site. In addition to our observation that MCG recruits larger number of neutrophils to the wound site, we noted that, in neutrophil-like differentiated HL-60 cells, MCG induced MCP-1 expression. This would suggest that via its activity on wound-site neutrophils, MCG would be able to mount a more inflammatory response by recruiting more macrophages to the wound site. Macrophages are beneficial to wound healing as long as inflammation resolves in a timely manner and does not end up being chronic. Analysis of the wound-edge tissue at multiple time points following wounding showed that MCG potently bolstered recruitment of macrophages to wound site. Importantly, such effect was accompanied with efficient resolution of inflammation as shown by the rapid return of macrophage count to control values. Taken together, MCG strengthened the inflammatory response following wounding by supporting neutrophil and macrophage chemotaxis.

Inflammation is widely recognized to be a major driver of angiogenesis. Peripheral blood monocyte subsets recruited into the wound site promote wound tissue vascularization. At the wound site, macrophages produce VEGF, which in turn supports endothelial cell proliferation. We noted induction of VEGF expression in the wound-edge tissue of MCG-treated wounds. Consistently, we noted higher abundance of vWF gene, a marker of endothelial cell in such treated wounds. Higher abundance of endothelial cells in MCG-treated wound-edge tissue was confirmed using immunohistochemical studies to detect endothelial cells. Endothelial cell proliferation, known to be induced by ECM component such as collagen, represents a major component of angiogenesis. Immunohistochemical studies showed that in wound tissue treated with MCG, endothelial cell proliferation was significantly higher. To test the functional significance of a
higher abundance of endothelial cells at the MCG-treated wound site, blood flow imaging was performed using a LASCI system. The LASCI is a high-resolution and high-contrast optical imaging technique often used to image blood flow dynamics in real time with excellent spatial and temporal resolution. Quantitative analysis of blood flow established that MCG treatment significantly improved blood flow to the wound tissue.

During the later remodeling stage of wound healing, appropriate collagen deposition is important because it increases the strength of the wound tissue resisting reopening caused by shear stress. In addition, it provides appropriate signaling cues to cells at the wound site to complete the healing process. We observed significantly increased collagen deposition during the later stages of MCG-treated wounds. Types I and III collagen are fibril forming interstitial collagens most abundant in the skin. Collagen type III (thin fibers) is laid down first early after injury, which supports the healing wound until it is replaced by stronger collagen type I (thick fibers) late during maturation. Increased collagen type I:III ratio is crucial for tensile strength of healing wounds. Several clinical studies have reported that decreased collagen I:III ratio was associated with postoperative recurrence of incisional hernia through surgical wounds. Also, impaired biomechanical properties of diabetic skin were associated with lowered collagen I and collagen I:III ratio. This explains the compromised mechanical stability of diabetic wounds to withstand traumatic injury. In this current work, MCG treatment significantly increased collagen I:III ratio on day 21 postwounding. In support of this observation, we noted that MCG induced the expression of type I collagen gene, demonstrating host skin response to the applied collagen dressing. Elongated rete ridges and elevated collagen I:III ratio predict improved ability of the MCG-treated wound to withstand shear stress and reopening.

In summary, the study presents first evidence from a preclinical setting explaining how a collagen-based dressing may improve wound closure by targeting multiple key mechanisms including a more robust transient inflammatory response followed by improved wound tissue vascularization and collagen deposition. The current findings warrant additional studies to determine whether the responses to the MCG are different from other modified or unmodified collagen-based products used in clinical setting.

ACKNOWLEDGMENT

We thank Prashant Sinha, MD, and Jennifer Dickerson, RVT, for assistance with the conduct of experiments. The work was supported by National Institutes of Health awards GM077185, GM069589, and DK076566. We thank Dr. Ed Stout and Ms. Angie McKee from Southwest Technologies for providing the dressing and unrestricted research development funding to The Ohio State University.

CONFLICT DISCLOSURE

The work was partly supported by Southwest Technologies Inc. by means of an unrestricted gift where the donor had no control over scientific experiments or reporting.


34. Browne CD, Hindmarsh EJ, Smith JW. Inhibition of endothelial cell proliferation and angiogenesis by orlistat, a fatty acid synthase inhibitor. FASEB J 2006; 20: 2027–35.


Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Figure S1. Representative MS/MS spectrum of peptide 1322VAGVANALAHIR 143 from hemoglobin subunit beta (bovine). The scan sequence of the mass spectrometer was based on the TopTen method; the analysis was programmed for a full scan recorded between 350–2000 Da, and a MS/MS scan to generate product ion spectra to determine amino acid sequence in consecutive instrument scans of the ten most abundant peak in the spectrum. Protein identifications were checked manually and proteins with a Mascot score of 50 or higher with a minimum of two unique peptides from one protein having a -b or -y ion sequence tag of five residues or better were accepted.

Figure S2. Experiment design. (A) Four sets with 8 excisional 3 mm punch wounds in each set were created on each side of the back of pigs (n = 4). One set of 8 wounds were collected at specified time points (days 3–21) postwounding. (B) Wounds on one side of the back were either treated with a modified collagen gel (MCG), followed by covering with Tegaderm (TD) or TD alone (control, contralateral side).