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Anchoring a Cytoactive Factor in a Wound Bed Promotes Healing

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Abstract

Wound healing is a complex process that requires the intervention of cytoactive factors. The one-time application of soluble factors to a wound bed does not maintain a steady, sufficient concentration. Here we investigate the benefits of anchoring a factor in a wound bed via a tether to endogenous collagen. We use a collagen mimetic peptide (CMP) as a pylon. The CMP binds to damaged but not intact collagen and thus localizes a pendant cytoactive factor in the regions of a wound bed that require intervention. As a model factor, we choose Substance P, a peptide of the tachykinin family that promotes wound healing. Using splinted wounds in *db*/*db* mice, we find that the one-time application of a CMP–Substance P conjugate enhances wound healing compared to unconjugated Substance P and other controls. Specifically, all 16 wounds treated with the conjugate closed more thoroughly and did so with extensive re-epithelialization and mitigated inflammatory activity. These data validate a simple and general strategy for re-engineering wound beds by the integration of beneficial cytoactive factors.

Keywords

collagen; extracellular matrix; *Mus musculus*; peptide; Substance P; synthetic biology

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1. Introduction

Slow-healing and chronic pathologic wounds have a major debilitating effect on the physical and mental health of patients, as well as being a significant drain on health care resources (Baum and Arpey, 2005; Broughton *et al.*, 2006; Gibran *et al.*, 2007; Gurtner *et al.*, 2008). Slow-healing wounds can manifest in victims of severe burn injuries and extensive physical trauma; chronic dysregulated wounds are associated with the corrupted wound beds imposed by diabetes and vascular abnormalities. Healing of these wound types is a complex process. Re-epithelialization, granulation tissue formation, collagen deposition, and contraction are critical phases of the healing process that determine the quality of healing, as well as the bias for wound repair or wound regeneration.

Previous efforts to promote wound healing have invoked exogenous cytoactive factors administered by subcutaneous or intraperitoneal injections (Barrientos *et al.*, 2008), as well as topical application (Murphy *et al.*, 2001; Lee *et al.*, 2002). The efficacy of these approaches is compromised by the natural lavation that rapidly dilutes and ultimately drains soluble molecules. We reasoned that anchoring a cytoactive factor on endogenous collagen, which comprises ¾ of the dry weight of human skin (Shoulders and Raines, 2009), could overcome this problem. Efforts have been made to immobilize molecules on Type I collagen by covalent coupling to its amino-acid side chains (Tiller *et al.*, 2001). This approach is, however, confounded by the heterogeneous functional groups on natural collagen and by collateral damage that necessarily ensues upon treating tissue with electrophilic alkylating or acylating agents.

We sought a strategy that surmounts these limitations without being derivative of any current practice in the clinical management of wounds. We were aware that collagen mimetic peptides (CMPs) can assemble spontaneously into a collagen triple helix under physiological conditions (Shoulders and Raines, 2009; Fields, 2010). We hypothesized that damaged collagen triple helices containing double-stranded regions could be targeted by a CMP, as we had shown that certain CMPs anneal to collagen *in vitro* and *ex vivo* but do not bind to intact triple helices (Hodges and Raines, 2005; Chattopadhyay *et al.*, 2012). Moreover, we suspected that the most damaged areas in a wound bed inherently provide the most binding sites for a CMP. Accordingly, we reasoned that a CMP could act as a pylon, anchoring a pendant cytoactive factor in the regions of a wound bed that require the most intervention (Figure 1).

To test our hypothesis, we sought a pendant cytoactive factor that does not need to be internalized into cells to stimulate proliferative activity in the wound bed. Among such factors is Substance P (SubP) (Reid *et al.*, 1993; Villablanca *et al.*, 1994; Nishida *et al.*, 1996; Nakamura *et al.*, 1997; Datar *et al.*, 2004). SubP is a well-characterized undecapeptide of the tachykinin family that is produced by sensory neurons, stored in the terminal end of unmyelinated cutaneous nerve fibers, and released upon noxious stimuli. Nerve fibers containing SubP have been identified in human skin and burn wounds in the vicinity of blood vessels and sweat glands (Dunnick *et al.*, 1996). After release from the sensory nerves, SubP binds to a specific cell-surface neurokinin receptor (NK-1). Upon release, SubP not only mediates vasodilation in early wound repair, release of histamine by mast cells, and

angiogenesis, but also leads to interaction with immunocompetent cells, including granulocytes and monocytes (Datar *et al.*, 2004). These effects are observed not only at the initial point of stimulus, but also in the surrounding area, as SubP shows proliferative activity on keratinocytes and fibroblasts (Kähler *et al.*, 1996). Thus, SubP participates in the vasodilation associated with an inflammatory response and plays a role in simulating proliferation of epithelial, vascular, and connective tissue (Nilsson *et al.*, 1985). Recent studies have demonstrated the efficacy of SubP for wound healing in mice, rats, and rabbits (Muangman *et al.*, 2009; Ishikawa *et al.*, 2013; Lee *et al.*, 2013; Kant *et al.*, 2013).

Here we report on the design and synthesis of a peptide in which SubP is linked covalently to a CMP. We then test this conjugate as an agent for healing cutaneous wounds in mice. The resulting data support a new molecular modality for the re-engineering of wound beds.

2. Materials and methods

2.1. Materials

SubP (acetate salt hydrate) was from Sigma–Aldrich (St. Louis, MO). Polyethylene glycol 8000 (PEG) from Fisher Bioreagents® (Fairlawn, NJ) and saline (bacteriostatic 0.9% w/v NaCl) from Hospira (Lake Forest, IL) were used to prepare 5% w/v PEG/saline as a vehicle.

Chemicals for peptide synthesis were of reagent grade or better, and were used without further purification. Anhydrous solvents were obtained from CYCLE-TAINER® solvent delivery systems (J. T. Baker, Phillipsburg, NJ). HPLC-grade solvents were obtained in sealed bottles (Fisher Chemical, Fairlawn, NJ). In all reaction mixtures having anhydrous solvents, glassware was either oven- or flame-dried.

2.2. Instrumentation

Semi-preparative HPLC was performed with a Varian Dynamax C-18 reversed-phase column. Analytical HPLC was performed with a Vydac C-18 reversed-phase column. Mass spectrometry was performed with an Applied Biosystems Voyager DE-Pro (matrix-assisted laser desorption/ionization) mass spectrometer from Life Technologies (Carlsbad, CA) in the Biophysics Instrumentation Facility at the University of Wisconsin–Madison.

2.3. Design of CMP–SubP peptide

In previous work, we showed that a 21-residue collagen mimetic peptide, $(ProProGly)_7$, does not form stable triple helices with itself but anneals strongly to type I collagen *in vitro* (Chattopadhyay *et al.*, 2012). In addition, we showed that this attribute can be used to anchor small-molecule fluorophores in wound tissue *ex vivo*, and that $(ProProGly)_7$ is not toxic to dermal fibroblast cells. Hence, we chose to employ $(ProProGly)_7$ as the CMP to effect our wound-healing strategy *in vivo*.

Next, we had to decide whether to conjugate SubP to the N terminus or the C terminus of $(ProProGly)_7$. SubP is known to induce cellular responses through distinct receptors that recognize different regions of the peptide, and both the N and the C terminus have been found to be active in this regard (Ananthanarayanan and Orlicky, 1992). Studies have established that the C-terminal region of SubP displays biological activities that are

mediated by the neurokinin receptor (Bury and Mashford, 1976; Iwamoto *et al.*, 1990). The C-terminal amide is also crucial to its biological activity, as the free acid is largely inactive in the chemotaxis of monocytes(Ruff *et al.*, 1985) and in its ability to bind to human T lymphocytes (Payan *et al.*, 1984). Accordingly, we synthesized a peptide with a C-terminal amide in which SubP was conjugated to the C terminus of $(ProProGly)_7$. This 32-residue peptide, CMP–SubP, has 16 proline residues and the amino-acid sequence: $(ProProGly)_7ArgProLysProGlnGlnPhePheGlyLeuMetNH_2$.

2.4. Peptide synthesis and purification

The CMP–SubP conjugate and the CMP were synthesized by solid-phase peptide synthesis using a 12-channel Symphony® peptide synthesizer from Protein Technologies (Tucson, AZ) at the University of Wisconsin–Madison Biotechnology Center. The CMP–SubP strand was initiated by coupling the C-terminal methionine residue to NovaPEG Rink Amide Resin (0.44 mmol/g, EMD Millipore, Darmstadt, Germany) and subsequent segment condensation using excess (5 equiv/coupling) FmocLeuOH, FmocGlyOH, FmocPheOH, FmocGln(Trt)OH, FmocProOH, FmocLys(Boc)OH, FmocArg(Pbf)OH, and FmocProProGlyOH, which was synthesized as described previously (Chattopadhyay *et al.*, 2012). The residues were converted to active esters by treatment with 1-hydroxybenzotriazole (HOBt, 3 equiv), *O*-benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate (HBTU, 3 equiv), and *N*-methylmorpholine (6 equiv). The ten other SubP amino acids were added to the C-terminal Met residue by extended (60-min) couplings. Seven FmocProProGlyOH tripeptides were added by normal (30-min) couplings. Fmoc-deprotection was achieved by treatment with piperidine (20% v/v) in DMF. The CMP itself was synthesized by SPPS on Fmoc-Gly-Wang Resin (0.4–0.7 mmol/g, 100 200 mesh, EMD Millipore) by the sequential coupling of FmocProOH and FmocProProGlyOH. CMP–SubP was cleaved from its resin using 92.5:5:2.5 trifluoroacetic acid/thioanisole/ethanedithiol (total volume: 3 mL), and the CMP was cleaved from its resin by using 95:2.5:2.5 trifluoroacetic acid/triisopropylsilane/water (total volume: 2 mL).

Both the CMP–SubP conjugate and the CMP were precipitated from *t*-butylmethylether at 0°C, isolated by centrifugation, and purified by semi-preparative HPLC using the following linear gradients: CMP–SubP, 10–90% B over 50 min, and the CMP, 5–85% B over 45 min; where solvent A was H_2O containing TFA (0.1% v/v) and solvent B was CH_3CN containing TFA (0.1% v/v). The CMP was readily soluble in H_2O, but the CMP–SubP conjugate required the addition of CH_3CN (12% v/v) to form a clear solution for HPLC analysis. Each peptide was judged to be >90% pure by HPLC and MALDI–TOF mass spectrometry: (*m*/*z*) $[M + H]^+$ calculated for CMP–SubP 3107, found 3108; (*m*/*z*) $[M + H]^+$ calculated for the CMP 1777, found 1777.

2.5. Design of wound model

As a wound host, we chose male mice that are homozygous for $Lepr^{db}$. Such *db*/*db* mice exhibit an impaired wound-healing response (Brem *et al.*, 2007), which increasing the sensitivity of wound-healing assays as well as clinical relevancy. The course of wound healing in these mice mimics that in humans with adult-onset type II diabetes mellitus (Greenhalgh, 2003).

As a wound type, we chose excisional wounds. These wounds heal from the margins, enabling the broadest assessment of the various parameters for wound healing, including re-epithelialization (Greenhalgh and Warden, 2001). We were aware, however, that the healing of mouse wounds is distinctly different from that of human wounds. In mice, contraction is the major mechanism of wound closure. In humans, re-epithelialization and granulation tissue-formation dominate (Greenhalgh and Warden, 2001). We devised a means to overcome this dichotomy. The use of splints around excisional wounds in *db*/*db* mice had been shown to allow healing by fibrovascular tissue formation and re-epithilialization, while minimizing the effects of contraction (Galiano *et al.*, 2004). In addition, a splinted wound model can provide two side-by-side wounds on the same mouse and facilitates the application of topical agents directly onto a wound bed. Accordingly, we used splinted wounds in *db*/*db* mice for our analyses.

2.6. Wound healing

Male mice (BKS.Cg-*Dock7*^{m} +/+ *Lepr*^{db}/J, Jackson Laboratories, Bar Harbor, ME) of age 8–12 weeks were housed in groups until the day of surgery. After surgery, mice were housed in separate cages. The mice were provided food and water *ad libitum*, as well as enrichment, and housed in a temperature-controlled environment with 12-h light and dark cycles.

On their day of surgery, mice were anaesthetized with Isoflurane (Abbott Laboratories, Abbott Park, IL) using an induction chamber. Buprenex® (buprenorphine hydrochloride, Reckitt Benckiser, Berkshire, UK), diluted in 0.9% w/v saline to a concentration of 0.01 mg/mL, was injected subcutaneously (0.4 mL/mouse) for pain management. Eyes were lubricated and hind nails clipped. The craniodorsal region was shaved using electric clippers, and the shaved area was scrubbed with alternating cotton swabs of 4% v/v chlorhexidine gluconate (Purdue Products, Stanford, CT) and sterile saline in circular strokes. Residual hair was removed.

Two identical wounds (8-mm o.d.) were created in the craniodorsal region of each *db*/*db* mouse (n = 8 mice/16 wounds per treatment regimen), as follows. O-Ring splints (15 mm o.d. × 11 mm i.d., 2-mm thickness, Carr® silicone O-rings from McMaster, Chicago, IL) were placed bilaterally in a symmetric arrangement using adhesive (Krazy Glue® Gel, Elmer's, Columbus, OH), and then secured to the skin with 8 interrupted sutures (5-0 nylon suture), encircling the splints with the knots (Galiano *et al.*, 2004). Wounds were created in the center of the splints using an 8-mm biopsy punch, and the wounding was completed using forceps and scissors to prevent the punch from lacerating the subcutaneous tissue.

The splinted wounds were treated topically with 25 μL of a solution of CMP–SubP (1 mM) dissolved in vehicle, which was 5% w/v PEG/saline. The mice were incubated for 30 minutes under anesthesia. The recovered mice were monitored over a period of 16 days. The 5% w/v PEG/saline vehicle was tested as a control, along with saline alone. The CMP (25 μL, 1 mM) and SubP (25 μL, 1 mM) in 5% w/v PEG/saline were also used as controls. The wounds were photographed, and the mice were allowed to recover on a warming pad.

Treated mice were monitored daily for behavioral changes, and their body weights were recorded on days 1, 3, 6, 9, 12, and 16. Splints were checked daily, and any broken or untied

suture was replaced according to the experimental protocol. During a 24-hour period, if only one suture was compromised, it was replaced with a new suture. If, however, two or more sutures were compromised during a 24-hour period, the wound was no longer considered splinted and was eliminated from the study.

On the last day of the experiment, wounds were photographed and the images were analyzed by calculating the wound area (mm^2) using ImageJ Software from the National Institutes of Health (Bethesda, MD). Wound closure was defined as the reduction in area between wound edges over the course of study and was reported as a percentage of the original wound size.

2.7. Wound harvesting

Histopathology cassettes were labeled for mouse and wound identification. Note cards (2½ cm^2) were fitted to the bottom of the histopathology cassettes and one edge was aligned with the cranial side of the harvested wound. On the final day of the experiment, mice were euthanized using Beuthanasia®-D (0.5 mL/mouse). Using a scalpel blade and scissors, 3 cm^2 of tissue was taken from the mouse, keeping the wound centered in the tissue section. Deep dissection was performed to harvest several layers of tissue deep in the wound. The square section of tissue was affixed to the note card. The cassettes were then closed and stored in formalin (10% v/v) at 1:10 tissue/fixative until histopathological analysis.

2.8. Histopathological analyses

After euthanasia, the entire wound bed as well as the intact skin margin greater than 5 mm was excised to the retro-peritoneum. The harvested tissue was then fixed in formalin (10% v/v) for at least 24 hours, and then sectioned through the center of the lesion. The center was marked with India ink prior to fixation. Routine paraffin processing was performed, and the tissue samples were sectioned serially at a thickness of 5 μm, making sure that the center of the lesion was included on the slide. The slides were stained with hematoxylin, eosin, and picrosirius red. A mounted digital camera (Olympus DP72, Melville, NY) was used to photograph the sections using light microscopy. Size of the wound, length of re-epithilialization, amount of fibrovascular proliferation in the dermis, and inflammatory response were measured as parameters to study wound healing on the slides containing the center of the lesion. Measurements were taken and analyzed using CellScience Dimension 1.4 image-analysis software from Olympus (Melville, NY). “Wound size” was defined as the area of the wound not covered by the advancing epithelial layer, and was calculated by measuring the distance between the opposite free edges of the wound. “Re-epithelialization” was defined by the length of the layer of proliferating keratinocytes covering the wounds area, and was calculated by measuring the distance between the free edge of the keratinocyte layer and the base where the cells were still associated with native dermal tissue. Both sides of the lesion were measured and the final result was the sum of the two measurements. For wounds that had undergone complete re-epithelialization, a single measurement was taken from base to base.

Fibrovascular dermal proliferation was measured by examining the picrosirius red-stained sections under polarized light, which highlights newly deposited dermal collagen (Junqueira *et al.*, 1979). The wound bed was designated with the image-analysis software, and the

amount of new collagen in the selected area was measured and expressed as a percentage of the total wound area to quantify “collagen deposition”.

The inflammatory response was assessed by a pathologist blinded as to the wound treatment using a semi-quantitative histopathological “inflammation score” ranging from 0 to 4, where 0 indicated no inflammation, 1 indicates 0-25% of the wound area being affected, 2 indicates 25–50% of the wound area being affected, 3 indicates 50–75% of the wound area being affected, and 4 indicates >75% of the wound area being affected. The inflammatory response was also categorized as ‘acute’ when more than 75% of the cells were neutrophils; ‘chronic active’ when there was a 1:1 ratio of neutrophils and mononuclear cells; and ‘chronic’ when >75% of the inflammatory cells were mononuclear.

2.9. Statistical analyses

Data were analyzed with a Mann-Whitney rank sum test, and statistical significance was set to $p < 0.05$. Analyses were executed with the program GraphPad Prism, Version 5.0 from GraphPad Software (La Jolla, CA).

2.10. Study approval

All animal experiments were approved by and performed in accordance with the guidelines and regulations set forth by the Institutional Animal Care and Use Committee of the University of Wisconsin–Madison.

3. Results

3.1. Wound healing by CMP–SubP

We generated eighty splinted wounds in forty *db*/*db* mice. We treated these wounds with CMP–SubP, SubP alone, the CMP alone, or vehicular controls. We found that a pendant CMP domain improves the efficacy of SubP in wound healing.

Sixteen days after treatment, 8-mm wounds treated with vehicle (saline or PEG/saline) showed considerable scab formation (>85% of the total wound area) and no discernible improvement in healing (Figures 2A and 2B). Most of the wounds treated with SubP or the CMP individually were still open on Day 16 (Figures 2C and 2D). In contrast, all of the wounds treated with CMP–SubP exhibited full closure (Figure 2E).

These visual observations were confirmed by detailed histopathological analyses of the eighty wounds (Figure 3). Lesions were present in all wounds, except those treated with CMP–SubP, which caused complete closure, minimal or no scab formation, and uniform fibrovascular proliferation (granulation tissue formation). Most notably, all wounds treated with CMP–SubP were closed completely (Figure 4A) with extensive re-epithelialization (Figure 4B). Collagen deposition was down-regulated in the presence of either SubP alone or CMP-SubP (Figure 4C). These data are in accord with a previous report of SubP causing a decrease in collagen biosynthesis, concomitant with a down-regulation of pro-α1(I) collagen mRNA and an increase of collagen biodegradation upon incubation with human lung fibroblast cells (Ramos *et al.*, 2007). In contrast, treatment with the CMP alone led to collagen formation comparable to that from the vehicular controls.

3.2. Inflammation by CMP–SubP

Inflammation is an inevitable consequence of tissue injury (Eming *et al.*, 2007). The inflammatory activity of SubP was mitigated in the CMP–SubP conjugate (Figure 4D). The observed inflammation was largely in the acute stage and was composed of polymorphonuclear leucocytes (PMNLs). Previous work indicated that phagocytic activity by the PMNLs and macrophages in this phase is stimulated by the four N-terminal residues of SubP (Bar-Shavit *et al.*, 1980). The migratory activity of the PMNLs also depends on the N-terminal residues, and probably occurs via a non-receptor-mediated mechanism, with the participation of basic groups in this region (Wiedermann *et al.*, 1989). The blocked N terminus of SubP in CMP–SubP might diminish its pro-inflammatory activity. The unblocked C terminus is, however, still capable of mediating fibroblast and monocyte migration into the wound bed (Bury and Mashford, 1976; Ruff *et al.*, 1985; Iwamoto *et al.*, 1990).

4. Discussion

Excisional wounds in mice close by contraction, wherein the wound margins are drawn towards the center of the wound. Contraction is driven by myofibroblasts (Cass *et al.*, 1997). Innervation can affect myofibroblasts, and its absence has been observed to delay wound closure, contraction, and healing (Smith and Liu, 2002). This effect can be reversed by administration of SubP (Khalil and Helme, 1996), which enhances the neuronal area and promotes cellular proliferation (Buttow *et al.*, 2003).

The topical application of SubP promotes wound healing (Murphy *et al.*, 2001; Lee *et al.*, 2002; Muangman *et al.*, 2009; Ishikawa *et al.*, 2013; Lee *et al.*, 2013; Kant *et al.*, 2013). We have demonstrated that this activity of SubP is enhanced by its being anchored in the wound bed via a pendant CMP that binds to damaged collagen (Figure 1). Upon treatment with a CMP–SubP conjugate, cutaneous wounds in *db*/*db* mice were closed more quickly with increased levels of epithelialization (Figures 2-4). This benefit accrued despite *db*/*db* mice having lower epidermal nerve profile count, area fraction, and area density compared to *db*/– mice (Underwood *et al.*, 2001), attributes that are detrimental to wound healing.

The formation of collagen was depressed in the presence of CMP–SubP. This outcome is likely to minimize the generation of scar tissue. By enhancing epithelialization and down-regulating collagen deposition, CMP–SubP could be especially useful for the treatment of smaller and surface wounds, where a bias towards wound regeneration over wound repair is desirable. Moreover, as SubP mediates vasodilation and neovascularization (Datar *et al.*, 2004), CMP-SubP could have utility in a variety of clinical contexts.

Interestingly, treatment with the CMP alone enhanced wound closure compared to the vehicular controls (Figure 4B). We hypothesize that the CMP could sequester newly formed collagen before triple helix formation is complete, enabling its faster assimilation into the wound bed. Indeed, the proliferation and differentiation of mesenchymal stem cells has been reported to be favored by the presence of a CMP (Lee *et al.*, 2008). Measurement of the new epithelial layer formed in the course of the treatment showed a mirroring trend, with our test peptide exhibiting a mean of ~7 mm of re-epithelialization in wounds that were originally 8

mm in diameter (Figure 4B). The extent of re-epithelialization induced by the CMP is less than that induced by SubP, indicating that wounds treated with the former peptide closed preferably due to contraction [which is not eliminated completely by the splints (Galiano *et al.*, 2004)] rather than enhanced keratinocyte proliferation.

We note that the use of CMP conjugates has several attractive attributes for wound repair and regeneration. The most damaged collagenous matrix in a wound bed naturally provides the most binding sites for a CMP conjugate (Figure 1). The ensuing self-assembly directs chemotherapeutic agents to the regions of a wound bed that require the most urgent care. Although growth factors have been applied topically to wounds, those growth factors are diluted and washed away, decreasing efficacy and increasing cost. In contrast, the CMP in our strategy acts as a pylon, anchoring the growth factor where it is most needed—the region of a wound bed that has suffered the most damage. Thus, we circumvent the need for repeated administration, as well as conventional but intrusive techniques such as subcutaneous injections. This advantage could be amplified by using 4-fluoroproline residues instead of proline residues in the CMP (Chattopadhyay *et al.*, 2012). In addition, we note that our approach is intrinsically theranostic—combining the potential for imaging and therapy (Kelkar and Reineke, 2011). Finally, we anticipate that the simplicity of our approach, which relies only on conjugation of a cytoactive factor to a CMP, provides an inroad for the care for wounds with synthetic biology (Lutolf and Hubbell, 2005).

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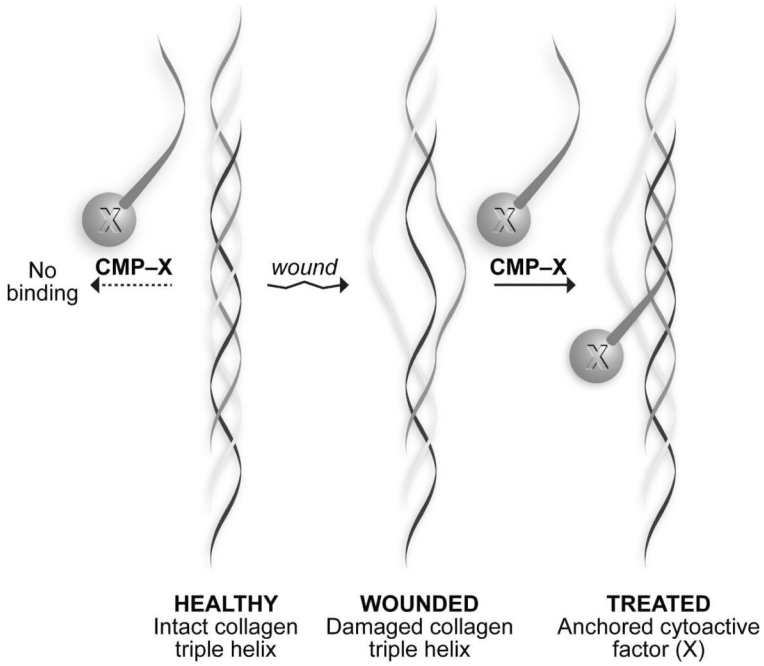

Figure 1.

Conceptual representation of a collagen mimetic peptide conjugate (CMP–X) annealed to a damaged collagen triple helix in a wound bed. “X” is a cytoactive factor that becomes anchored in the wound bed.

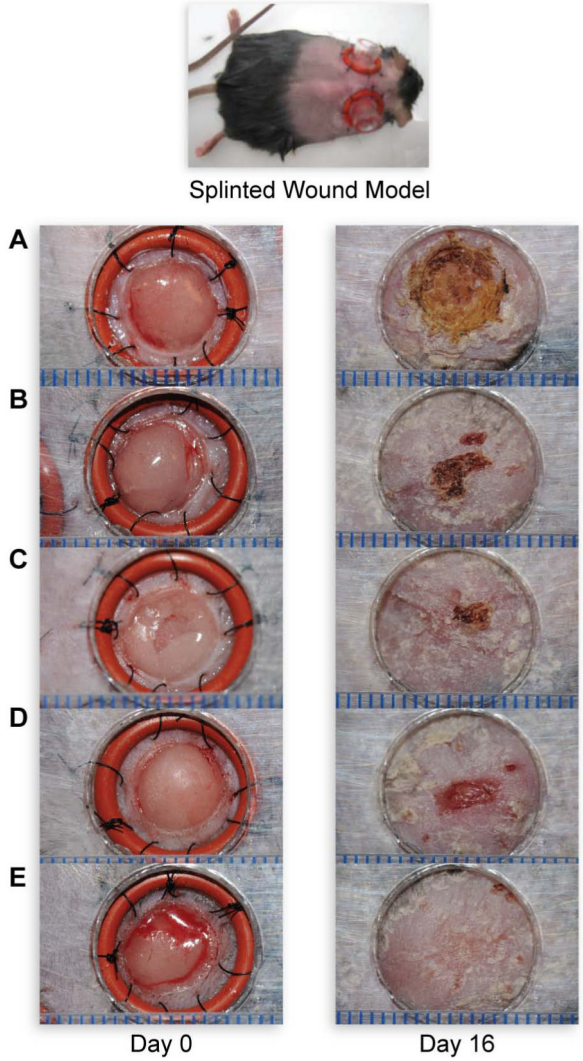

Figure 2.

Photographs depicting the effect of SubP-immobilization on splinted mouse wounds. Representative images are from Day 0 immediately post-treatment, or Day 16 after removal of the splints but before euthanasia. Wounds (8-mm o.d.) were treated with (A) saline, (B) PEG (5% w/v) in saline, (C) SubP (25 nmol) in 5% w/v PEG/saline, (D) CMP (25 nmol) in 5% w/v PEG/saline, or (E) CMP–SubP (25 nmol) in 5% w/v PEG/saline.

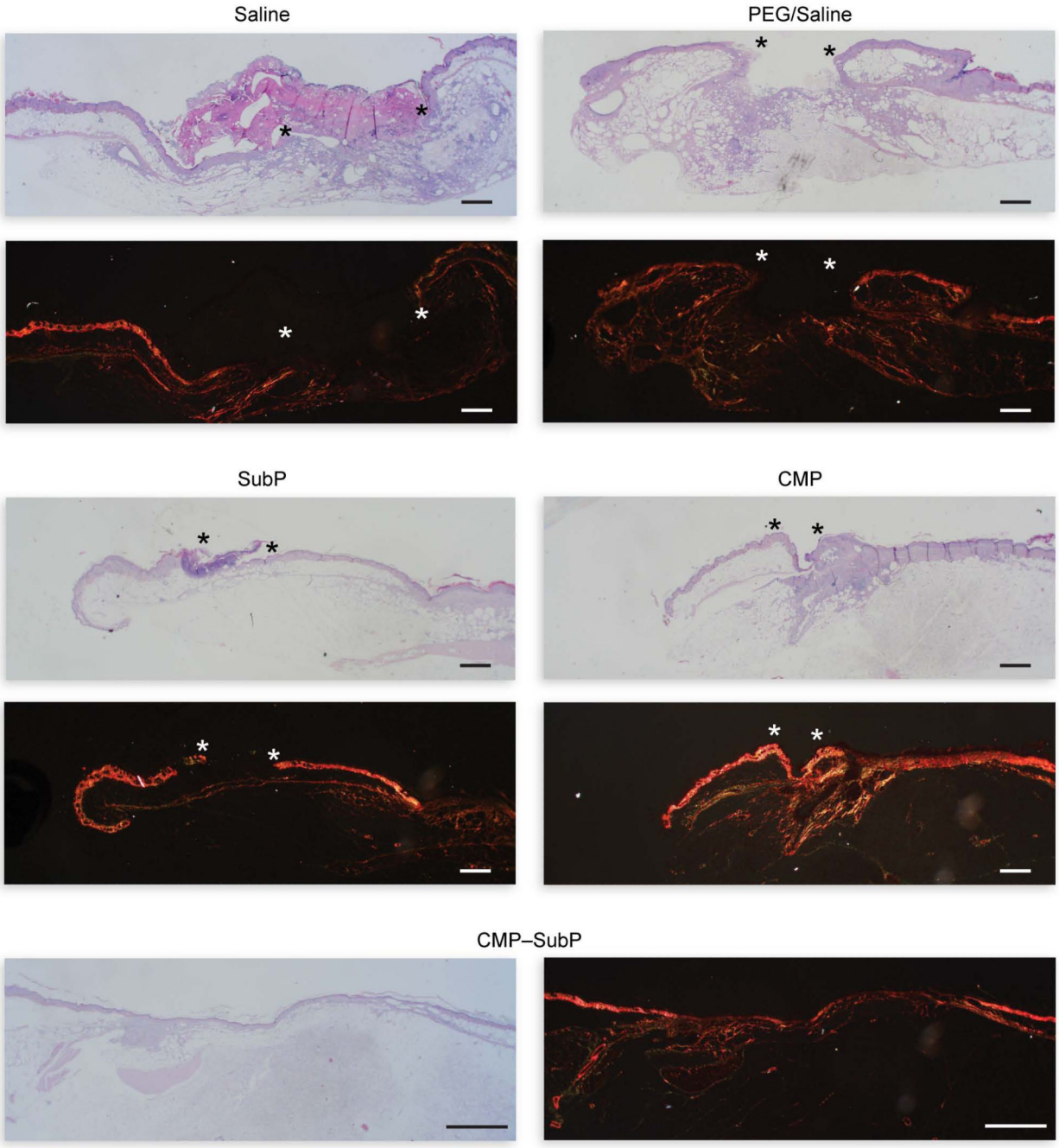

Figure 3.

Effect of SubP-immobilization on wound histology. Representative histological images are from Day 16 after treatment as described in Figure 2. Wounds were stained with hematoxylin + eosin and viewed under bright-field light (top or left panels; 2× objective or 4× objective for CMP–SubP), and stained with picrosirius red and viewed under polarized light (bottom or right panels; 2× objective or 4× objective for CMP–SubP). Wound edges are indicated by "*" symbols. Scale bars: 500 μm.

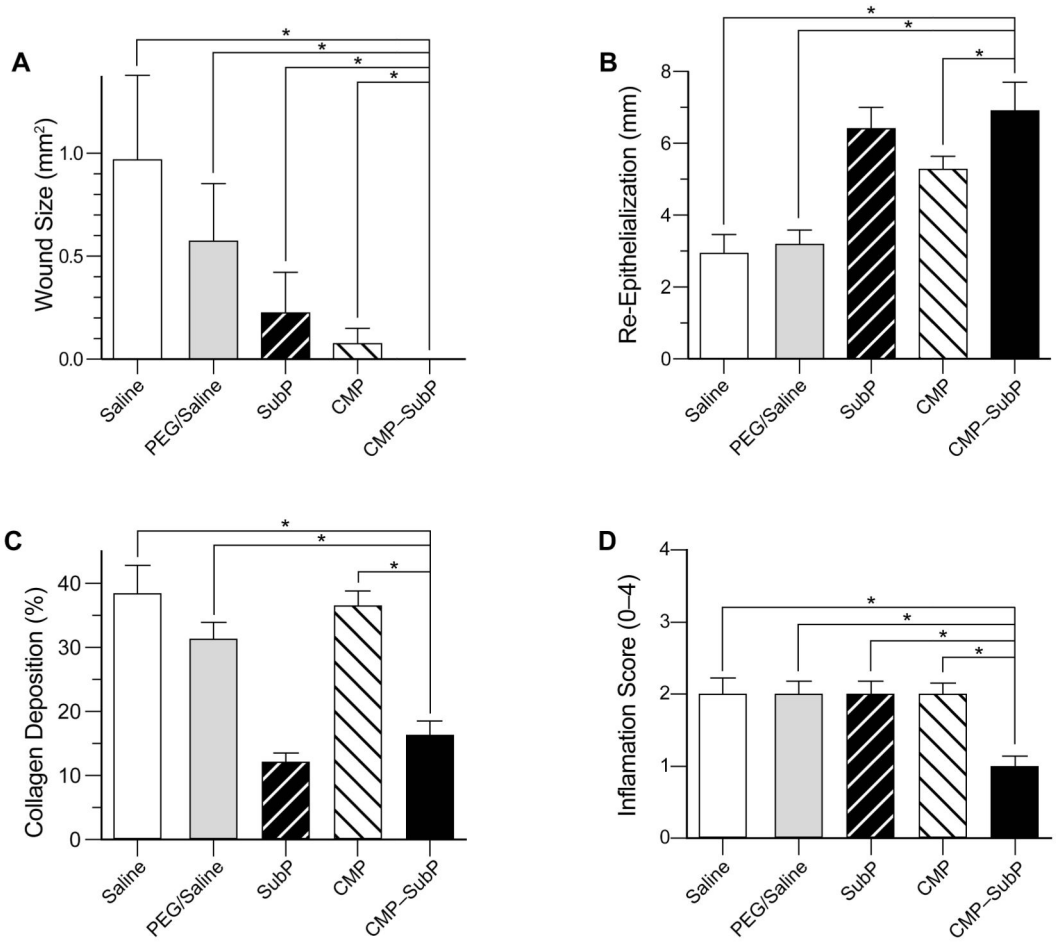

Figure 4.

Effect of SubP-immobilization on wound healing. Data are from Day 16 after treatment as described in Figure 2. (A) Mean size of wounds. (B) Re-epithelialization of wounds. (C) Collagen deposition as a percentage of the total area of the wound. (D) Inflammation in wounds on scale of 0–4. Data are the mean ± SE from histopathological analyses of 80 wounds (= 5 regimens × 16 wounds/regimen); asterisks (*) indicate significant differences ($p < 0.05$).