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Natural healing-inspired collagen-targeting surgical protein glue for accelerated scarless skin regeneration

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ABSTRACT

Skin scarring after deep dermal injuries is a major clinical problem due to the current therapies limited to established scars with poor understanding of healing mechanisms. From investigation of aberrations within the extracellular matrix involved in pathophysiologic scarring, it was revealed that one of the main factors responsible for impaired healing is abnormal collagen reorganization. Here, inspired by the fundamental roles of decorin, a collagen-targeting proteoglycan, in collagen remodeling, we created a scar-preventive collagen-targeting glue consisting of a newly designed collagen-binding mussel adhesive protein and a specific glycosaminoglycan. The collagen-targeting glue specifically bound to type I collagen in a dose-dependent manner and regulated the rate and the degree of fibrillogenesis. In a rat skin excisional model, the collagen-targeting glue successfully accelerated initial wound regeneration as defined by effective reepithelialization, neovascularization, and rapid collagen synthesis. Moreover, the improved dermal collagen architecture was demonstrated by uniform size of collagen fibrils, their regular packing, and a restoration of healthy tissue component. Collectively, our natural healing-inspired collagen-targeting glue may be a promising therapeutic option for improving the healing rate with high-quality and effective scar inhibition.

1. Introduction

Aberrations in highly orchestrated wound healing processes cause fibrotic tissues, which is known as a scar [1]. Excessive scarring after deep dermal injuries, including trauma, burn, and surgery, creates concerns for patients regarding significant physical dysfunction and psychological and/or aesthetic damage. Although it is obviously ideal that scar management should occur at the time of surgery to reduce subsequent scar formation, unfortunately, the current clinical scar managements, including invasive and non-invasive methods, focus on the treatment of established scars [1,2]. Besides, despite numerous current researches with advanced tissue engineering strategies for effective wound healing, there are still limitations such as unclear action mechanism, poor integration with host tissue, and side effects from high doses and administration frequency for desired efficacy [3,4]. With a better perception of abnormal structural and composition changes of the extracellular matrix (ECM) involved in scarring, novel targeting factors within ECM can be discovered for the development of scar-preventive therapeutics.

Collagen, the most abundant ECM structural protein, holds a vital clue for normal tissue development, maintenance, and regeneration as a substrate for cell attachment and a repository for various bioactive molecules [5,6]. Collagen is strictly organized via the self-assembly process known as fibrillogenesis, and the reorganization during wound healing can alter not only its cellular phenotype and cell-matrix interactions but also the physicochemical properties and structural integrity of healed tissues, which ultimately defines tissue-specific functions [5,6]. In impaired scars, collagen aligns in a single direction parallel to a basement membrane with loosely packed fibrils of uncontrolled diameter, unlike the basket weave-like pattern with normal fibril characteristics in fully healed tissue [2]. Therefore, eliciting normal collagen reorganization can be an important target to improve the healing rate and quality.

Decorin, a representative collagen-modulator, is known to

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regulate linear and lateral fibril growth for collagen packing and organization by binding type I collagen fibril via its collagen-binding domain and glycosaminoglycan (GAG) side chain [7]. In pathologic tissues such as hypertrophic scars, its remarkable reduction is correlated with dysfunctional regulation in collagen reorganization [8]. Indeed, a decorin-deficient animal model exhibited abnormal collagen architecture, skin fragility, and impaired healing [9]. However, there is a realistic limit for the use of native decorin for clinical studies due to its uneconomical purification from animal sources, difficult synthesis, and the fact that not all residues of decorin are responsible for the interaction with collagen [10]. In this context, a practical option seems to produce a simplified functional biomolecule by mimicking decorin as a model.

Mussel adhesive protein (MAP) secreted from the marine mussel has several captivating features, including strong underwater adhesion and biocompatibility [11,12]. A recent report also suggested that the adhesion between MAP and type I collagen could occur through extensive H-bonding, π-cation, and electrostatic interactions [13]. Previously, redesigned recombinant MAP was successfully mass-produced in a bacterial system with a simple purification process and shown to be biocompatible, biodegradable, and strongly but flexibly adhesive to various surfaces [14–16]. Moreover, the recombinant MAP exhibited desired biologic functions such as improved cellular behaviors via a peptide-fusion strategy [17,18] and facile surface functionalization based on electrostatic interactions [19,20]. The recombinant MAP also enabled tight wound sealing and rapid healing in a rat skin incisional wound model via *in situ* light-activated gelation [21].

In this work, to create a novel natural healing-inspired scar-preventive surgical glue, we first constructed three fusion MAPs containing different collagen-binding peptides (CBPs) derived from decorin or collagen I platelet receptor, and selected the most functional fusion MAP based on collagen-binding ability and fibrillogenesis tests. Then, we hypothesized that the electrostatic assembly consisting of cationic collagen-targeting MAP and anionic dermatan sulfate (DS), the GAG side chain of decorin, could behave similarly to decorin by directly targeting type I collagen, modulating collagen reorganization, and providing sufficient biochemical cues for scarless wound healing as an advanced regenerative template (Fig. 1). Herein, we aimed not only to demonstrate positive influences of MAP on overall wound healing phases, but also to highlight how the bioinspired collagen-targeting system can contribute to the alleviation or prevention of scar formation.

2. Materials and methods

Construction of expression vectors for collagen-targeting fusion MAPs containing CBPs. *Escherichia coli* TOP10 (Invitrogen) was used for recombinant plasmid construction. *E. coli* BL21(DE3) (Novagen) was used as a host strain for expressing recombinant proteins. The cloning scheme used to produce collagen-targeting fusion MAPs containing CBPs was approximately identical to the previous report [18]. In brief, through polymerase chain reaction (PCR), each type of CBPs, including LRELHLNNN (DEC), RRANAALKAGELYKCILY (CPR) and RRANAALKAGELYKSILYGC (mCPR), was added to the C-terminus of hybrid type MAP, fp-151 [14]. Each fusion protein was named MAP-DEC, MAP-CPR, and MAP-mCPR, respectively. Herein, the following primer sets were used to amplify encoding DNA for each fusion protein; MAP-DEC, forward 5′-GATCCGCCAAACCTTCTTACCCACCGAC-3′ and reverse 5′-CTCGAGTCAGTACAGGATGCATTTATACAGTTCGCCCGCTTTCAGGGCCGCGTTCGCGCGACGAAGCTT-3′; MAP-CPR, forward 5′-GATCCGCCAAACCTTCTTACCCACCGAC-3′ and reverse 5′- CTCGAGTCAATTGTTGTTCAGATGCAGTTCACGCAGAAGCTTCTTGTACGTTGGAGGATAAG-3′; MAP-mCPR, forward 5′-GATCCGCCAAACCTTCTTACCCACCGAC-3′ and reverse 5′- CTCGAGTCAGCAGCCGTACAGGATGCTTTTATACAGTTCGCCCGCTTTCAGGGCCGCGTTCGCGCGACGAAGCTTCTTGTACGTTGGAGGATAAG-3′. Each amplified fragment was then inserted into plasmid vector pET22(b)+ (Novagen). The nucleotide sequences of the inserted genes were verified by direct sequencing.

Preparation of collagen-targeting fusion MAPs containing CBPs. Recombinant fusion MAPs were produced and purified in *E. coli* as described previously [14]. Before they were used for *in vivo* applications, the undesirable impurities of recombinant MAPs such as lipopolysaccharides (LPS), endotoxins from the Gram-negative bacterial cell wall, were sufficiently removed according to the previously reported purification steps [16]. The expression and purity of each sample were assessed by 12% (wt/vol) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified samples were freeze-dried and stored at −80 °C for further analyses. Protein concentration was determined using the Bradford assay (Bio-Rad) with bovine serum albumin (BSA) (Promega) as a protein standard.

Collagen-binding ability test. The binding activities of collagen-targeting fusion MAPs to type I collagen were tested by microplate assays [22]. Each MAP or BSA dissolved in 5% (vol/vol) acetic acid was diluted in coating buffer (50 mM sodium carbonate/bicarbonate buffer; pH 9.4). The solutions were then incubated to immobilize them to a high-binding 96-well black/clear-bottom plate (Costar) at final concentrations of 2, 5, and 10 nmol per well for 8 h at 37 °C. BSA-coated wells were used as a negative control. Coated-wells were then washed three times with phosphate-buffered saline (PBS), and then BSA was incubated at 10 mg/mL in PBS for 1 h at 37 °C to inhibit non-specific binding. After washing with PBS, fluorescein isothiocyanate (FITC)-labeled collagen solution (Sigma) was diluted to 0.02 mg/mL in PBS containing 5 mg/mL of BSA and incubated at room temperature for 30 min. Unbound collagen-FITC was removed by washing three times with PBS, and fluorescence was measured on a spectrophotometer. The amount of the bound protein was quantified by subtracting the unbound supernatant fluorescence from the total fluorescence. Relative binding percentage of each protein was calculated relative to that (100% binding) of MAP-mCPR at 10 nmol per well. At least four independent samples were averaged to obtain each measurement.

***In vitro* collagen fibrillogenesis test.** To compare the effects of collagen-targeting fusion MAPs on collagen fibrillogenesis, turbidities of mixtures containing collagen and each sample were monitored at 313 nm as described previously [23]. The pre-gel solution was prepared by mixing stock collagen solution with 10× PBS, 1 M NaOH, and 1× PBS on ice to a final concentration of 4 mg/mL and pH of 7.4. At this time, a protein sample was added as a part of the final 1× PBS addition at a 1:1 M ratio of collagen: sample, followed by incubation on ice for 30 min before initiating fibrillogenesis by warming to 37 °C. Samples were added to each well of a 96-well plate at 4 °C, and measurements were taken at 3-min intervals for up to 3 h using a spectrophotometer at 37 °C. Moreover, to confirm the effect of DS on fibrillogenesis, additional tests including sole DS and DS addition were performed using the same procedure. All measurements were performed in triplicate.

SDS-PAGE analysis of type I collagen binding. Collagen fibrils were formed by mixing 3.5 μL of collagen (2.9 μg/μL in 12 mM HCl) with 21.5 μL of 10 mM HCl and 25 μL of TES buffer (60 mM TES, 20 mM Na_2HPO_4, and 0.56 M NaCl, pH 7.6). After incubating at 37 °C for 1 h, the fibrils were recovered as a pellet by centrifugation. Fibrils were recovered again by centrifugation after incubation at 37 °C for 1 h. To evaluate the role of DS in binding of MAP-mCPR to collagen, the fibril pellets were incubated in MAP-mCPR solution with or without DS at the same molar concentration as collagen overnight at 37 °C. The fibrils were recovered by centrifugation, and