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Cell recruiting chemokine-loaded sprayable gelatin hydrogel dressings for diabetic wound healing

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ABSTRACT

In this study, we developed horseradish peroxidase (HRP)-catalyzed sprayable gelatin hydrogels (GH) as a bioactive wound dressing that can deliver cell-attracting chemotactic cytokines to the injured tissues for diabetic wound healing. We hypothesized that topical administration of chemokines using GH hydrogels might improve wound healing by inducing recruitment of the endogenous cells. Two types of chemokines (interleukin-8; IL-8, macrophage inflammatory protein-3α; MIP-3α) were simply loaded into GH hydrogels during *in situ* cross-linking, and then their wound-healing effects were evaluated in streptozotocin-induced diabetic mice. The incorporation of chemokines did not affect hydrogels properties including swelling ratio and mechanical stiffness, and the bioactivities of IL-8 and MIP-3α released from hydrogel matrices were stably maintained. *In vivo* transplantation of chemokine-loaded GH hydrogels facilitated cell infiltration into the wound area, and promoted wound healing with enhanced re-epithelialization/neovascularization and increased collagen deposition, compared with no treatment or the GH hydrogel alone. Based on our results, we suggest that cell-recruiting chemokine-loaded GH hydrogel dressing can serve as a delivery platform of various therapeutic proteins for wound healing applications.

Statement of Significance

Despite development of materials combined with therapeutic agents for diabetic wound treatment, impaired wound healing by insufficient chemotactic responses still remain as a significant problem. In this study, we have developed enzyme-catalyzed gelatin (GH) hydrogels as a sprayable dressing material that can deliver cell-attracting chemokines for diabetic wound healing. The chemotactic cytokines (IL-8 and MIP-3α) were simply loaded within hydrogel during *in situ* gelling, and wound healing efficacy of chemokine-loaded GH hydrogels was investigated in STZ-induced diabetic mouse model. These hydrogels significantly promoted wound-healing efficacy with faster wound closure, neovascularization, and thicker granulation. Therefore, we expect that HRP-catalyzed *in situ* forming GH hydrogels can serve as an injectable/sprayable carrier of various therapeutic agents for wound healing applications.

1. Introduction

Diabetes mellitus, a complex metabolic disorder, is a significant health problem severely affecting millions of people over the world [1,2]. One of the most prevalent chronic complications associated with diabetes is diabetic foot ulcer (DFU) that results from impaired wound healing [3]. In general, optimum wound healing comprises a series of well-orchestrated cellular and biomolecular events in several overlapping phases, including inflammation, cell migration/proliferation, and tissue remodeling [4]. Various types of cells participate in each of the phases of wound healing, including inflammatory cells, fibroblasts, endothelial cells, keratinocytes, and mesenchymal stem cells (MSCs) [5]. In particular, it is suggested that the infiltration of MSCs *via* chemotaxic signaling into wound sites facilitates wound-healing processes [6,7]. MSCs have

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a high degree of plasticity and they are capable of differentiating into several other cell lineages that contribute to tissue repair [8,9]. They also regulate the wound environment by secreting various cytokines and growth factors to promote re-epithelialization/angiogenesis and collagen deposition [10]. However, diabetes mellitus inhibits MSCs invasion into wound sites during the initial stages of wound healing due to insufficient chemotactic response, consequently resulting in impaired wound healing [11,12].

One strategy to compensate for the limited cellular signals in the diabetic wound environment involves topical administration of endogenous MSCs-attracting therapeutic proteins (*e.g.*, chemokines and growth factors) [13–15]. However, direct treatment with these proteins has been found to be of limited efficacy in inducing the sequences of wound repair due to rapid denaturation and loss of bioactivity in the pathological environment [3]. To overcome these limitations, combination with biomaterial carriers that can efficiently deliver the bioactive agents to the target site has been proposed and extensively examined over several decades [16,17].

Polymeric hydrogels have been extensively used as promising dressing materials for diabetic wound treatment, because they provide the moist and occlusive environments proven to facilitate wound healing [18]. Particularly, *in situ* forming hydrogel dressings can be easily applied on the top of wound sites *via* injection or a spray. They offer several advantages for wound healing applications, including closer contact with surrounding tissues, good tissue conformity by accommodating large and irregular wound defects, and enhanced patient compliance [17,19]. Moreover, a variety of water-soluble therapeutic agents can be simply loaded inside their matrix, which serves as an implantable platform for local delivery, providing advanced functionality for more effective DFU treatment [20]. Among various *in situ* gelling systems, enzymatic cross-linking has received increasing interest for preparation of injectable or sprayable hydrogels. Some researchers have demonstrated the desirable features of horseradish peroxidase (HRP)-catalyzed *in situ*-forming hydrogels for wound healing applications [21–24]. These hydrogels showed controllable physico-chemical properties (*e.g.*, hydrogel stiffness, proteolytic degradation, and gelation rate), and they enhanced wound-healing efficacy with faster wound closure, neovascularization, and thicker granulation.

In our previous study, we developed HRP-triggered *in situ* cross-linkable gelatin-hydroxyphenyl propionic acid (GH) hydrogels as an injectable carrier for tissue engineering and regenerative medicine [25,26]. We have also identified two chemokines (interleukin-8; IL-8, macrophage inflammatory protein-3α; MIP-3α) that possess the ability to recruit bone marrow-derived mesenchymal stem cells (BMSCs) for articular cartilage repair [27]. It is well known that these chemokines play a crucial role in the migration of inflammatory cells as well as MSCs [28]. In addition, IL-8 is also known to be a potent promoter of angiogenesis by enhancing the survival and proliferation of endothelial cells [29]. Therefore, we hypothesized that local administration of these chemokines to wound defects using *in situ* forming hydrogels might improve wound healing of diabetic ulcer by enhancing MSC migration into the defect sites.

The goal of this study was to develop sprayable gelatin-hydroxyphenyl propionic acid (GH) hydrogels that can deliver MSC-attracting chemotactic cytokines to induce enhanced diabetic wound healing. Two types of chemokines (IL-8 and MIP-3α) were loaded within the GH hydrogels during *in situ* HRP-catalyzed cross-linking reaction, and we investigated their release profiles from the hydrogel matrices and bio-stabilities. Thereafter, the efficacy of IL-8- or MIP-3α-incorporated GH hydrogels in inducing *in vivo* wound healing was evaluated by assessing wound closure and re-epithelialization rates, degree of neovascularization, and collagen contents in a streptozotocin-induced diabetic mouse model.

2. Materials and Methods

2.1. Materials

Gelatin (type A from porcine skin), 3-(4-hydroxyphenyl) propionic acid (HPA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), horseradish peroxidase (HRP; 250–330 U/mg), hydrogen peroxide (H_2O_2; 30 wt% in H_2O), and bovine serum albumin (BSA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Dimethylformamide (DMF) was obtained from Junsei (Tokyo, Japan). All chemicals and solvents were used without further purification.

2.2. Synthesis of GH conjugates

GH conjugates were synthesized as previously described [30]. Briefly, HPA was activated with EDC and NHS in a cosolvent of water and dimethylformamide (volume ratio of 3:2), and the solution was added to preheated gelatin solution. After 24 h of reaction at 40 °C, the resulting solution was dialyzed against deionized water, filtered, and lyophilized to obtain GH conjugates. The chemical structure of the GH conjugates was characterized by ^{1}H NMR (AS400, Oxford Instruments, UK) and UV analysis. To quantitatively measure the HPA content of the GH polymers, the optical density of aqueous GH solution (1 mg/mL) was recorded at 275 nm using a UV visible spectrophotometer (V-750, Jasco, Japan), and then the phenolic content was determined using a calibration curve of tyramine hydrochloride solution.

2.3. Preparation of chemokine-loaded GH hydrogels

The GH hydrogels were prepared by cross-linking of phenol moieties in the presence of HRP and H_2O_2 *via* enzyme-catalyzed oxidative reaction [25]. After hydrogel formation, we did not wash to remove the HRP enzyme for the next experiments. A solution of GH polymer (6.25 wt%) dissolved in Dulbecco's phosphate-buffered saline (DPBS) was mixed with IL-8 (5 μg/mL) or MIP-3α (10 μg/mL), and then the mixtures were divided into two aliquots. HRP (0.03 mg/mL) was added to one aliquot and H_2O_2 (0.1 wt%) was added to another aliquot (volume ratio of GH:IL-8 or MIP-3α:HRP/H_2O_2 = 8:1:1). The GH hydrogels were formed by mixing the two aliquots at room temperature. The final experimental conditions for the preparation of chemokine-loaded GH hydrogels are provided in Table 1.

2.4. Characterization of chemokine-loaded GH hydrogels

2.4.1. Rheological analysis

The storage modulus (G′) of the chemokine-loaded GH hydrogel was measured using a rheometer (Advanced Rheometer GEM-150-050, Bohlin Instruments, USA) in oscillation mode. To the bottom plate, 200 μL of hydrogels containing IL-8 or MIP-3α were applied, and the upper plate (diameter = 25 mm) was subsequently lowed to a gap of 0.5 mm. The measurement at a frequency of 0.1 Hz and a strain of 0.01% was performed until the storage modulus of hydrogels reached a plateau value.

2.4.2. Swelling ratio

To measure the swelling degrees of GH hydrogels with or without chemokine (IL-8 or MIP-3α), 400 μL of the GH hydrogels were prepared in a Teflon mold ($10 \times 10 \times 0.5$ mm^3), and incubated in 5 mL of DPBS at 37 °C for 3 days. After the weight of swollen gels