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Visible light-cured glycol chitosan hydrogel dressing containing endothelial growth factor and basic fibroblast growth factor accelerates wound healing *in vivo*

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ABSTRACT

Wounds that heal with excessive scar formation result in poor functional and aesthetic outcomes. To address this, in our study, visible light cured glycol chitosan (GCH) hydrogels containing endothelial growth factor (EGF) and basic fibroblast growth factor (bFGF) were prepared (GCH-EGF, GCH-FGF and GCH-EGF/FGF) and evaluated their efficacies on the improvement of wound healing *in vivo*. *In vitro* release test showed that the growth factors were released in a sustained manner along with initial burst for 24 h. *In vitro* cell proliferation assay of L-929 mouse fibroblast cell line resulted in the superior ability of GCH-EGF/FGF on the rate. *In vivo* results demonstrated that the growth factor loaded GCHs further enhanced wound healing compared with GCH. In particular, GCH-EGF/EFG showed the most remarkable wound healing effect among the samples.

Introduction

During the wound healing process, several types of growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), are secreted, and these growth factors are very helpful in wound healing [1]. Recently, the combined use of growth factors have received much attention as a fascinating method of accelerating wound healing, along with minimizing scar formation [2,3]. Among the growth factors, a combination use of EGF and FGF can be a good combination for accelerated wound healing due to their some merits. EGF is related to dermal wound healing through stimulation, proliferation and migration of some cells including keratinocytes, endothelial cells and fibroblasts, leading to facilitation of dermal regeneration [4]. Among the FGF family, basic FGF (bFGF) is involved in granulation formation, reepithelialization, and matrix formation and remodeling [5]. However, to date, he combined use of EGF and bFGF in wound healing has been scarcely reported.

Despite the merits of the growth factors, the use of growth factors alone has some limitations in clinical applications, because topical administration results in insufficient chronic wound healing due to early inactivation of growth factors in the wound site [6]. In addition, the half-life of growth factors is too short for them to exert their biological activities effectively when applied in free forms [6].

Polymeric hydrogel systems are capable of overcoming the drawbacks of growth factors by encapsulation [7]. They have been developed as prime candidates for wound healing due to their hydrophilic nature and soft tissue-like properties [7]. In addition, polymeric hydrogels provide a moist wound healing environment

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and aid in the removal of dead tissue from the wound site during the healing process [7]. Polymeric hydrogels also play significant roles in preventing the invasion of bacteria and oxygen to the wound, thereby providing a barrier against infections [7].

In our previous study, visible light-cured glycol chitosan (GCH) hydrogel systems have been prepared as wetted wound dressing agents, along with the encapsulation of biomolecules such as growth factors and drugs [3,8]. Owing to their water-solubility, GCH-based hydrogels can be easily prepared in aqueous solution. In addition to the facile preparation, GCH-based hydrogels can maintain a moist environment and protect against external contaminants because of the antibacterial effect of GCH. Some physicochemical methods have been reported for the preparation of polymeric hydrogels. Among them, visible light irradiation is one of most fascinating methods, because it is safer than ultraviolet (UV), enables easy control of temporal and spatial reaction kinetics, and rapidly induces a reaction via a single step process under mild conditions [9].

Herein, we prepared advanced wound dressings, based on visible light-cured GCH hydrogels containing a combination of EGF and bFGF, for accelerated wound healing. The wound healing effect was compared with that of GCH hydrogel, and GC hydrogels containing EGF or bFGF alone using mouse excisional wound model *in vivo*.

Experimental

Materials

Glycol chitosan (GCH) and glycidyl methacrylate (GM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Riboflavin 5′-monophosphate sodium salt (riboflavin) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were obtained from Gibco™ (Thermo Fisher Scientific, Waltham, MA, USA). 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) was supplied by Wako Pure Chemical Industries (Osaka, Japan). Dialysis tubes (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) were used for purification. The mouse fibroblast L-929 cell line was supplied by the Korean Cell Line Bank (Seoul, Republic of Korea). Eagle's minimal essential medium, fetal bovine serum (FBS), and penicillin & streptomycin (Life Technologies; Grand Island, NY, USA) were used for cell culture. All chemicals were used as received without further purification.

Preparation of EGF and/or FGF-loaded GCH hydrogels

GM/GCH was prepared as reported previously [3,8]. GCH hydrogels containing EGF (10 μM), bFGF (10 μM) or EGF/bFGF (10/10 μM) were prepared as follows: EGF, bFGF or EGF/bFGF was added to a solution of GM/GCH in phosphate-buffered saline (PBS, pH 7, 1 mL) and gently shaken to mix homogeneously. After adding riboflavin (12 μM), the mixtures were photo-cured for 20 s with a visible irradiator (430–485 nm, 2100 mW/cm^2, light-emitting diode (LED) curing light, Forshan Keyuan Medical Equipment Co., Ltd., Guangdong, China). The hydrogels were designated as GCH-EGF, GCH-FGF and GCH-EGF/FGF, respectively.

Characterizations of GCH, GCH-EGF, GCH-FGF and GCH-EGF/FGF

The storage and loss moduli of the hydrogel precursor solutions before and after visible light irradiation as a function of frequency were measured by an AR 2000 EX rheometer (TA instrument, New Castle, DE, USA) set with a cone and plate geometry of 4 cm in diameter and at cone angle of 1°. The surface and cross-sectional morphologies after visible light irradiation were observed by scanning electron microscopy (SEM; CX-200TM; COXEM Ltd., Daejeon, Republic of Korea). For the observation, the hydrogels were attached on metal mounts using carbon tape, gold-coated using an ion sputter-coater (COXEM Ltd., Daejeon, Republic of Korea) and imaged at 20.0 kV with a magnification of ×500. After subjecting each solution (370 μL) into the rotational cone and plate at controlled shear rates, the moduli were measured at 37 °C ranging from 0 Hz to 100 Hz. At determined time intervals (0, 4, 7, 10 and 14 days), the hydrogels immersed in water were extracted and washed with distilled water three times. After removing the water from the hydrogel surfaces, they were weighed. Swelling ratio was calculated by the ratio of the swollen weight to the initial weight of the hydrogels. The degradation behavior was analyzed at the determined time intervals by measuring the degradation ratio, which is expressed as the ratio of the remaining weight to the initial weight of the hydrogels.

Release behavior of EGF and/or bFGF

At predetermined time intervals (1, 3, 6 and 12 h, and 1–3, 5, 10, 15, 20, 25 and 30 days), the solutions (2 mL) were collected in the hydrogels incubated with 3 mL PBS (pH 7.4) in an incubator set at 37 °C with 100 rpm and fresh PBS (2 mL) was added to each hydrogel. The solutions were measured using a microplate reader at wavelength of 450 nm for the detections of EGF and bFGF.

L-929 cell proliferation assay

For sterilization, the hydrogel precursor solutions added to 1 mL syringes were maintained in an autoclave set at 121 °C for 15 min. After visible light irradiation, the hydrogels (50 μL) were placed into 96-well plates and L-929 cells (1×10^4 cells/well) were seeded on each hydrogel and incubated for 1, 3 and 7 days. At the determined time intervals, Cell Counting Kit-8 (CCK-8) reagent (100 μL) was added to the wells and incubated for an additional 2 h. The optical density of the supernatants was measured using a microplate reader (SpectraMax® i3, Molecular Devices, CA, USA) at 450 nm.

In vivo *wound healing study*

Animal experimental protocols were approved by the Chonnam National University Animal Research Committee. Fifteen Balb/C mice (20 g, n = 6) were used for the *in vivo* full thickness skin wound healing study. For anesthetization, Ketara® (50 mg/kg) and Rompun® (5 mg/kg) were used after shaving the back of the mice. Two circular wound defects (6 mm) were created per each mouse. Each hydrogel was placed on the defects randomly. Image J program was employed to measure the changes in the wound areas at the determined time intervals (0, 4, 7, 10 and 14 days). After day 14, the mice were euthanized by exposure to 100% CO_2. The surrounding skin and muscle including the defects were extracted and fixed in 10% neutral buffered formalin. Tissue samples were embedded in paraffin and sectioned at 3 μm thickness for hematoxylin and eosin (H&E) and Masson's Trichrome (MT) staining. Each staining was carried out according to the manufacturer's protocols. The stained slides were visualized by fluorescence microscopy (AX70, TR-62A02, Olympus, Tokyo, Japan).

Statistical analysis

All quantitative data were expressed as the mean ± standard deviation. Statistical analysis was performed with one-way analysis of variance (ANOVA) using SPSS software (SPSS Inc.,

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