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Controlled-release of epidermal growth factor from cationized gelatin hydrogel enhances corneal epithelial wound healing

Kuniko Hori [a,*], Chie Sotozono [a], Junji Hamuro [a], Kenta Yamasaki [a], Yu Kimura [b], Makoto Ozeki [b], Yasuhiko Tabata [b], Shigeru Kinoshita [a]

[a] *Department of Ophthalmology, Kyoto Prefectural University of Medicine, Hirokoji, Kawaramachi, Kamigyoku, Kyoto 602-0841, Japan*
[b] *Department of Biomaterials, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan*

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Abstract

We designed a new ophthalmic drug-delivery system for epidermal growth factor (EGF) from the biodegradable hydrogel of cationized gelatin. We placed a cationized gelatin hydrogel (CGH) with incorporated ^{125}I-labelled EGF in the conjunctival sac of mice and measured the residual radioactivity at different times to evaluate the *in vivo* profile of EGF release. Approximately 60–67% and 10–12% of EGF applied initially remained 1 and 7 days after application, respectively; whereas EGF delivered in topically applied solution or via EGF impregnation of soft contact lenses disappeared within the first day. We also placed CGH films with 5.0 μg of incorporated EGF on round corneal defects in rabbits to evaluate the healing process using image analysis software and to assess epithelial proliferation immunohistochemically by counting the number of Ki67-positive cells. The application of a CGH film with incorporated EGF resulted in a reduction in the epithelial defect in rabbit corneas accompanied by significantly enhanced epithelial proliferation compared with the reduction seen after the topical application of EGF solution or the placement of an EGF-free CGH film. The controlled release of EGF from a CGH placed over a corneal epithelial defect accelerated ocular surface wound healing.

Keywords: Controlled release; Corneal wound healing; Epidermal growth factor; Cationized gelatin hydrogel; Hydrogel degradation

1. Introduction

Growth factors (GF) are relevant bioactive proteins that play a vital role in regulating cell functions and maintaining tissue homeostasis; a wide variety of GF are involved in the wound-healing process of the ocular surface [1,2]. An enormous number of studies have revealed that topical treatment with GF, such as epidermal growth factor (EGF) [3–7], keratinocyte growth factor [8], acidic and basic fibroblast growth factor [9,10], and nerve growth factor [11] enhanced the wound-healing effect in primate models and in clinical trials, suggesting promising therapeutic efficacy of GF in ocular surface diseases. As these findings elucidated more extensively the clinical usefulness of GF, a system to deliver protein drugs has become more and more needed. Compared to conventional synthetic low molecular weight drugs, proteins are more unstable during their storage and administration, and they are susceptible to denaturation during the drug formulation process [12–14].

This has hampered the ocular therapeutic application of GF, and eye drops, the simplest formulation for ophthalmic administration, have remained the standard mode of drug delivery. However, rapid pre-corneal loss requires the frequent instillation of highly concentrated drug solutions to achieve drug contact with the cornea for periods long enough to obtain therapeutic efficacy [15–17]. To overcome the low bioavailability of drugs in conventional eye drops, various drug delivery systems have been explored. Viscous vehicles such as ointments and polymeric hydrogels improve drug bioavailability moderately [15–19]. Hydrogel soft contact lenses (SCL) [20–23] have been used as a pre-corneal drug reservoir system and collagen shields [24–26] have been studied as an alternative to provide higher and sustained drug concentrations for up to several hours. As available drug delivery systems focused on the delivery of low molecular weight drugs rather than protein drugs, we set out to design a finely tuned system to deliver GF to the ocular surface.

* Corresponding author. Tel.: +81 75 251 5577; fax: +81 75 251 5663.
E-mail address: khori@ophth.kpu-m.ac.jp (K. Hori).

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We previously explored the use of biodegradable gelatin hydrogels for the controlled release of GF [27–29] and succeeded in enhancing the *in vivo* biological activity of GF in angiogenesis [30,31], osteogenesis [32,33], and adipogenesis [34]. From the release system, biologically active GF were released as a result of biodegradation of hydrogel as the release carrier.

In the current study we investigated whether the gelatin hydrogel could be used for the controlled release of GF by examining the release profiles of GF incorporated into the gelatin hydrogel and accelerated ocular surface wound-healing effect. Because it enhances epithelial wound healing [3–7], we used EGF as a representative GF. We prepared anionized and cationized derivatives of acidic- and basic-type gelatins, respectively, and various hydrogels by cross-linking gelatin samples. We then impregnated the hydrogels with EGF to obtain the hydrogel incorporating EGF and examined the *in vitro* and *in vivo* EGF release profiles. To evaluate their therapeutic efficacy, we applied the hydrogel incorporating EGF to rabbit corneal epithelial defects, measured the decrease in the size of the defects, and quantified epithelial proliferation by counting the number of Ki67-positive cells.

2. Materials and methods

2.1. Preparation of gelatin derivatives

Gelatin was chemically derivatized by introducing functional groups into acidic-and basic-type gelatin (isoionic point (IIP) 5.0 and 9.0, Nitta Gelatin Co., Osaka, Japan) to obtain anionized or cationized gelatin derivatives, respectively. For anionization, succinic anhydride was conjugated to the amino groups of acidic gelatin side-residues to obtain a succinylated derivative of gelatin [35]. For another anionization, 2.606 g of sulfoacetic acid (5 ml) were added to 45 ml of 4 wt.% acidic-type gelatin solution in phosphate-buffered saline (PBS); then the pH was adjusted to 5.0. After adding 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, the reaction was continued at 37 °C for 18 h; this was followed by dialyzing against milli-Q purified water for 48 h at room temperature (RT). The dialyzed solution was freeze-dried to obtain a sulfoethylated gelatin derivative. For cationization, various amounts of ethylenediamine were conjugated to the amino groups of basic gelatin side-residues to obtain cationized gelatin (CG) [36–38]. The CG was named according to the molar ratio of ethylenediamine added to the carboxyl groups of gelatin (E0, E0.5, E3, E10, and E50). Using the 2,4,6-trinitrobenzene sulfonate (TNBS) method [39], the percent introduced for each derivative was calculated as the percentage increase or decrease of the amino groups in cationized or anionized gelatins. The IIP of the derivatives was measured by the JIS PAGI method [40]. Briefly, 1 wt.% of gelatin derivative solution was passed through ion exchange column (cationic (DOWEX 50W-X8) and anionic (DOWEX 1-X8) exchange resins were mixed) at 40 °C and the pH of the elution was measured with pH meter (HORIBA D-22) at 40 °C.

2.2. Preparation of gelatin hydrogels

2.2.1. Preparation of freeze-dried gelatin hydrogel blocks and SCL

The gelatin hydrogel blocks were prepared by chemical cross-linking of gelatin [35,38]. The freeze-dried gelatin hydrogels were cut into blocks of 2.0 and 1.0 mg for *in vitro* and *in vivo* release experiments, respectively. Commercially available SCL (Precision UV®) were rinsed 3 times with milli-Q, freeze-dried, and cut into 4.0- and 2.0-mg blocks.

2.2.2. Preparation of thin CG hydrogel (CGH) films

Thin CGH films were prepared by air-drying and dehydrothermal cross-linking of CGH. Aqueous solutions of 5 wt.% CG (1000 μl for mouse — and 250 μl for rabbit experiments) were cast into $2 \times 2\text{-cm}^2$ molds, air-dried at 4 °C, and then cross-linked in a vacuum oven at 160 °C for 54 h. The resulting CGH films (0.025 mm in thickness) were cut into 2.0-mg pieces for the mouse experiments; they were punched out with a trephine to obtain 9-mm round pieces for the rabbit experiments.

2.2.3. Measurement of gelatin hydrogel water content

The water content of the gelatin hydrogel (weight ratio of water in the hydrogel to the wet hydrogel) was calculated from the hydrogel weight before and after 24-h swelling in milli-Q at 37 °C [35].

2.3. Estimation of in vitro EGF release

Human recombinant EGF (PeproTech EC Ltd., London, UK) was radiolabelled using the chloramine-T method [41]. After dropping 10 μl of ^{125}I-labeled EGF solution onto freeze-dried gelatin hydrogel blocks and SCL, they were left overnight at 4 °C to obtain gelatin hydrogels and SCL with incorporated ^{125}I-labeled EGF (^{125}I-EGF-gelatin hydrogels, ^{125}I-EGF–SCL). They were then agitated at 37 °C in 1 ml of PBS. The PBS supernatant was removed and replaced at 30, 60, 120, and 360 min or at 24 and 48 h with an identical volume of fresh PBS solution. The radioactivity in each supernatant was measured on a gamma counter (ARC-301B, Aloka, Tokyo, Japan) to obtain the EGF release profiles over time ($n=3$, at each time point).

2.4. Animal experiments

All animal experiments were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research; all experimental procedures were pre-approved by the Committee for Animal Research of Kyoto Prefectural University of Medicine and Kyoto University. We used 5-6-week-old female ddY mice in our *in vivo* EGF release and CGH degradation experiments and female Japanese white rabbits, each weighing 1.5–2.0 kg, in the wound-healing experiments.

2.5. Estimation of in vivo EGF release

We used radiolabelled EGF to obtain the *in vivo* EGF release profiles in the conjunctival sac of mice. The animals were