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Efficacy of gelatin gel sheets sustaining epidermal growth factor for murine skin defects

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ARTICLE INFO

Article history:
Received 22 June 2015
Received in revised form
5 October 2015
Accepted 18 November 2015
Available online 24 November 2015

Keywords:
Gelatin gel sheet
Sustained release
Epidermal growth factor
Wound healing
Murine skin defects

ABSTRACT

Background: Epidermal growth factor (EGF) plays an important role in wound healing. However, EGF must be applied daily due to rapid inactivation *in vivo*. We investigated the sustained release of EGF from gelatin gel sheets (GGSs) and the efficacy of GGSs impregnated with EGF for promoting wound healing.

Materials and methods: GGSs impregnated with EGF were prepared by cross-linking via glutaraldehyde to gelatin solution containing EGF. The sustained release of EGF and the bioactivity of released EGF were evaluated. Then, three kinds of GGSs containing NSS (normal saline solution; NSS group), 2.5 μg of EGF (EGF-L group), or 25 μg of EGF (EGF-H group) were applied to full-thickness skin defects created on the backs of mice. The wounds covered with polyurethane film without GGS were used as a control (PUF group). The wound area, neoepithelium length, regenerated granulation tissue, and newly formed capillaries were evaluated.

Results: EGF was sustained and released from GGS as it degraded. The bioactivity of released EGF was confirmed. EGF-L group promoted the neoepithelium length, regenerated granulation tissue, and newly formed capillaries compared with those in the PUF and NSS groups. The area of regenerated granulation tissue in the NSS group (week 1: $2.6 + 0.2$ mm^2, week 2: $2.8 + 0.3$ mm^2) was larger than that in the PUF group (week 1: $0.6 + 0.1$ mm^2, week 2: $1.0 + 0.1$ mm^2). The area of newly formed capillaries in the EGF-L group ($9967 + 1903$ μm^2) was larger than that of the EGF-H group ($3485 + 1050$ μm^2).

Conclusions: GGSs impregnated with EGF-L showed promising results regarding wound healing.

1. Introduction

The process of wound healing is dynamic and interactive, and it involves three phases as follows: inflammation, tissue formation, and tissue remodeling [1]. Various growth factors, such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and transforming growth factor-β (TGF-β), play important roles in wound healing at each phase [2,3].

EGF is a polypeptide of 53 amino-acids which was first isolated from the mouse submaxillary gland by Cohen [4–6]. EGF stimulates the proliferation and migration of epidermal cells, fibroblasts, and endothelial cells and promotes

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0022-4804/$ – see front matter © 2016 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jss.2015.11.027

epidermal regeneration, angiogenesis, and granulation tissue formation [7,8]. The efficacy of EGF in wound healing has been reported experimentally and clinically in acute wounds [9–11], chronic wounds [12–14], and burn wounds [15,16]. EGF is commercially available in Korea (as Easyef; Daewoong Pharmaceutical Co, Ltd, Seoul, Korea), China, and some countries in South America.

EGF is metabolized, quickly diffuses after local administration, and is shortly inactivated [17,18]. Easyef is recommended to be applied twice a day, which is burdensome for both the patients and the medical staff. As mentioned previously, a sustained release system of EGF that can reduce the frequency of application is thus desirable in clinical practice.

Gelatin is derived from collagen, has been used in medical and pharmaceutical products, and its safety has been proved through its long-term clinical use. Gelatin has also been reported to sustain EGF [10], bFGF [19–22], and TGF-β [22,23] depending on its isoelectric point (IEP) and release them according to its biodegradation. This sustained system of gelatin with EGF, bFGF, and TGF-β has been reported to be effective in wound healing [10,20,22] and tissue regeneration [24,25].

In this study, we prepared gelatin gel sheets (GGSs) by cross-linking the gelatin solution using glutaraldehyde (GA). Bioactive proteins, such as growth factors, can be impregnated into the gelatin solution before cross-linking. We hypothesized that GGSs were able to achieve the sustained release of EGF and that GGSs impregnated with a proper dosage of EGF could accelerate the wound healing process. We confirmed the sustained release of EGF from GGSs and the bioactivity of the released EGF and explored the efficacy of this system in the wound healing process using a murine model of skin defects.

2. Materials and methods

2.1. Ethics statement and experimental animals

The mice were maintained in the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. The number of animals used in this study was kept to a minimum, and all efforts were made to minimize animal suffering in compliance with the protocols established by the university's Animal Research Committee. Our experimental protocol was approved by the Animal Research Committee (permit number: Med Kyo 13,141).

Isolation and culture of human dermal fibroblasts were approved by the Kyoto University Graduate School and Faculty of Medicine, Ethics Committee (permit number: E568). A patient and her father were informed of this study and agreed to offer her tissue specimen. Written informed consent was obtained from the patient and her father.

2.2. Preparation of GGS impregnated without and with EGF

Gelatin powder (AP270; Nippi Inc, Tokyo, Japan), an extract obtained from porcine skin by an acidification process with a molecular weight of 68,800 and IEP of 9.0, was dissolved in distilled water (Otsuka Pharmaceutical Co, Ltd, Tokyo, Japan), and 20 weight percent (wt%) gelatin solution was prepared. GGSs were prepared as follows: 400 μL of 20 wt% gelatin solution and 50 μL of normal saline solution (NSS: Otsuka Pharmaceutical Co, Ltd) were mixed in a 13-mm-diameter iron ring (Yahata Neji Co, Ltd, Aichi, Japan) and stirred by a pipet tip. Then, 50 μL of 2% GA solution (Wako Pure Chemical Industries, Ltd, Osaka, Japan) was added as a cross-linker and stirred at 37°C for 10 s to cross-link the gelatin solution, thus forming NSS-GGS. The two GGSs impregnated with different concentrations of EGF were prepared in the same process. Recombinant human EGF (Easyef) was purchased from Daewoong Pharmaceutical Co, Ltd. Four hundred microliters of 20 wt% gelatin solution and 50 μL of 0.005% EGF (Easyef) or 50 μL of 0.05% EGF (Easyef) were mixed in a 13-mm-diameter iron ring (Yahata Neji Co, Ltd) and stirred by a pipet tip. Thereafter, 50 μL of 2% GA solution was added as a cross-linker and stirred at 37°C for 10 s to cross-link the gelatin solutions, forming EGF-L GGS and EGF-H GGS. An EGF-L GGS contains 2.5 μg of EGF and an EGF-H GGS contains 25 μg of EGF.

2.3. Sustained release of EGF from GGS and its bioactivity

2.3.1. EGF release from GGS

EGF-L GGSs ($n = 36$) were prepared. Three GGSs were used to measure its dry weight. The remaining GGSs ($n = 33$) were individually placed into 15-mL test tubes (Thermo Fisher Scientific K.K., Osaka, Japan). Each GGS was immersed with 2.5 mL of phosphate-buffered saline (PBS; Life Technologies Co, Ltd, Tokyo, Japan) and incubated in 5% CO_2, 100% humidity at 37°C. Then, each tube was shaken at 60 rpm by an *in vitro* shaker (Shake-XR; Taitec Co, Ltd, Saitama, Japan). Two hundred microliters of solution and GGSs from three tubes were collected at 1, 3, 5, 8, and 24 h after shaking. After incubation for 24 h in PBS, the solution in the remaining 18 tubes was discarded and 2.5 mL of 8 U/mL collagenase L solution (Nitta gelatin Inc, Osaka, Japan) was added. Then, at 1, 3, 5, 8, 24, and 48 h after changing to collagenase L solution, 200 μL of collagenase solution and GGSs from the three tubes were collected. GGSs immersed in the collagenase solutions were immediately washed with 10 mL of distilled water to stop the enzymatic reaction. The concentration of EGF in the solution was measured by enzyme-linked immunosorbent assay kits (Human EGF immunoassay kit; R&D Systems, Inc, Minneapolis) according to the manufacturer's instructions. The optical densities of each solution were measured by a spectrophotometer (VersaMax; Molecular Devices, Lilac, CA) at a test wavelength of 450 nm and a reference wavelength of 540 nm. The sum of the average amount of EGF released in PBS for 24 h and the average amount of EGF released in the collagenase solution 48 h after was expressed as 100%, and the amount of released EGF at each point was expressed as the percentage of the total EGF. GGSs were dried using a freeze-dryer (VD-250R; Taitec Co, Ltd) and the weight of GGSs was measured. The average dry weight of three GGSs before immersing in PBS was expressed as 100%, and the degradation rate of the gelatin gel at each point was expressed as a percentage.

2.3.2. Bioactivity of EGF released from GGS

Dulbecco modified Eagle medium (DMEM, D6429; Sigma–Aldrich Co, LLC, Tokyo, Japan) with 0.5% fetal bovine serum