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Efficacy of the dual controlled release of HGF and bFGF impregnated with a collagen/gelatin scaffold



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ABSTRACT

Background: We previously developed collagen/gelatin sponges (CGS) able to sustain and release basic fibroblast growth factor (bFGF) and reported that this CGS impregnated with bFGF promoted dermis-like tissue formation. We herein confirmed the single-sustained release of hepatocyte growth factor (HGF) and the dual sustained release of HGF and bFGF from CGSs, and explored its efficacy using a murine model of skin defects.

Materials and methods: The sustained release of HGF alone and both HGF and bFGF from CGSs were evaluated in vitro. CGSs (8 mm in diameter) impregnated with normal saline solution (NSS) (NSS group), HGF solution (10 or 50 $\mu\text{g}/\text{cm}^2$) (HGF-L or HGF-H group), bFGF solution (7 $\mu\text{g}/\text{cm}^2$) (bFGF group), or HGF (10 $\mu\text{g}/\text{cm}^2$) and bFGF (7 $\mu\text{g}/\text{cm}^2$) solution (HGF + bFGF group) were implanted into full-thickness skin defects on the backs of mice. The wound area, neoeepithelium length, dermis-like tissue formation and newly formed capillaries were evaluated.

Results: The single release of HGF and the dual release of HGF and bFGF from CGSs were confirmed. At week 1, the wound closure and neoeepithelium length were promoted in the HGF-L group compared with the NSS group. At week 2, the wound closure, neoeepithelium length, dermis-like tissue formation and newly formed capillary formation were promoted in the bFGF and HGF + bFGF groups compared with the NSS and HGF-H groups. Newly formed capillary formation was superior in the HGF + bFGF group compared with the bFGF group.

Conclusions: The dual release of HGF and bFGF from CGS was a promising treatment for full-thickness skin defects.

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Introduction

Wound healing is an evolutionarily conserved, complex multi-cellular process that is coordinated by numerous cells, such as

keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets.¹ This complex process is regulated by a complex signaling network harmonized by numerous cytokines such as growth factors and chemokines.² Therefore, it is important to

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reproduce this process by releasing the optimal dose of various growth factors at the optimal time to promote wound healing.

We developed a bilayered acellular artificial dermis (Pel-nac: Gunze Ltd, Tokyo, Japan) with an upper silicone layer and a lower layer made of collagen sponge, which has been used for the treatment of full-thickness skin defects caused by severe burns, trauma, or tumor excision for more than 15 years.³⁻⁸ However, some issues still remain to be solved in the clinical use of artificial dermis. One major issue is its weakness against infection, so it cannot be easily applied for the treatment of chronic ulcers, such as diabetic ulcers.^{9,10} Therefore, we developed collagen/gelatin sponges (CGS) that could sustain and release positively charged growth factors such as basic fibroblast growth factor (bFGF)¹¹ and platelet-derived growth factor (PDGF).¹² We previously showed that CGSs impregnated with bFGF promoted angiogenesis and dermis-like tissue formation in decubitus ulcers on diabetic mice.¹³

Hepatocyte growth factor (HGF) was originally discovered as a mitogenic factor of rat hepatocytes in primary culture.¹⁴⁻¹⁷ HGF is reported to promote the proliferation of vascular endothelial cells and keratinocytes and promote angiogenesis and epithelization. HGF is also reported to prevent fibrosis, which leads to scar contracture.¹⁸⁻²⁰ Regarding the wound healing effect of HGF, HGF is reported to promote angiogenesis, granulation formation, and epithelization both in acute wounds^{21,22} and in chronic wounds²³ by its topical application.

In this study, we focused on the synergistic effect of bFGF and HGF in a sustained manner. bFGF is a potent angiogenic factor, however, it does not accelerate epithelization in higher dosage.^{13,24} HGF promotes both angiogenesis and epithelization. HGF is positively charged, therefore, we hypothesized that HGF could be impregnated into CGSs and released in a sustained manner to accelerate angiogenesis and epithelization. We evaluated the dual controlled release of HGF and bFGF from CGSs and then evaluated those effects in the healing process of murine full-thickness skin defects.

Materials and methods

Ethics statement and experimental animals

We maintained the animals in the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. We kept the number of animals used in this study to a minimum, and made all possible efforts 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 14,569).

Preparation of CGS

We used gelatin isolated from pig dermis, with an isoelectric point (IEP) of 5.0 and a molecular weight of 99,000 (Nippi Inc, Tokyo, Japan), and atelocollagen isolated from pig tendons, with an IEP of 8.5 and a molecular weight of 300,000 (Nitta Gelatin Inc, Osaka, Japan). CGS was prepared as described

previously.^{11,24} The CGS with a gelatin concentration of 10 weight percent (wt%) of the total solute was prepared by mixing 3 wt% gelatin solution with 0.3 wt% collagen solution. We then spread a thin layer of silicone paste (Shin-Etsu Chemical Co, Ltd, Tokyo, Japan) onto a polyester mesh. Before the silicone paste dried, the top of the CGS was attached to the silicone paste-covered polyester mesh. As the silicone paste dried, it formed a sheet that adhered to the CGS. CGSs of 1.5 mm and 3 mm thicknesses were prepared. CGSs of 3 mm thickness were used for *in vitro* experiments and CGSs of 1.5 mm thickness were used for mice experiments, because CGSs of 3 mm thickness were designed for clinical treatment of human skin defects. Murine skin is thinner compared with human skin, thus CGSs of 1.5 mm thickness were used for mice experiments in this study.

In vitro release study of HGF and bFGF from CGS

We previously confirmed that CGS could sustain bFGF and release it according to its biodegradation in a sustained manner *in vivo* and *in vitro*.^{11,13} In this study, we investigated whether CGS could sustain HGF alone or both HGF and bFGF simultaneously and release them in a sustained manner using the same method described in our previous study.¹² Recombinant HGF, kindly supplied from Tsubouchi H and Shimizu A,^{16,25} and recombinant bFGF (Fiblast Spray, Kaken Pharmaceutical Co Ltd, Tokyo, Japan) were used in this study. Regarding the dosage of HGF, it is reported that the topical application of 7 $\mu\text{g}/\text{cm}^2$ of HGF on six consecutive days to full-thickness skin defects of diabetic mice promoted granulation tissue formation, wound angiogenesis, and epithelization.²³ Regarding the dosage of bFGF, we previously reported that CGS impregnated with 7 $\mu\text{g}/\text{cm}^2$ bFGF accelerated dermis-like tissue formation in the skin defects of normal mice.²⁴ In this study, we evaluated the impregnation of HGF to CGS and its release at a dosage of 10 $\mu\text{g}/\text{cm}^2$ and dual sustaining and release of HGF at 10 $\mu\text{g}/\text{cm}^2$ and bFGF at 7 $\mu\text{g}/\text{cm}^2$.

Single sustain and release of HGF

Thirty-three CGSs measuring 10 $\times$ 15 mm in size and 3.0 mm in thickness were prepared, and each CGS was initially weighed. CGSs were impregnated with 10 $\mu\text{g}/\text{cm}^2$ of HGF as follows: we prepared a normal saline solution (NSS: Otsuka Pharmaceutical Co, Ltd, Tokyo, Japan) containing HGF at a concentration of 0.1 $\mu\text{g}/\mu\text{L}$ and applied 150 μL of the HGF solution to CGS. After overnight incubation at 4°C, each CGS was transferred into a 15-mL test tube (Thermo Fisher Scientific K.K., Yokohama, Japan) and immersed in 2.5 mL of phosphate-buffered saline (PBS; Thermo Fisher Scientific K.K.). Then, CGSs were shaken at 60 rpm using an *in vitro* shaker (Shake-XR: Taitec Co, Ltd, Saitama, Japan) at 37°C. Three CGSs were taken from the tube just after immersion to measure the dry weights before biodegradation. Then, three CGSs and 200 μL of the solution from the three tubes were collected at 1, 3, 5, 8, and 24 h after immersion. After incubation for 24 h, the solutions in the remaining 15 tubes were discarded and 2.5 mL of 4 U/mL collagenase L solutions (Nitta Gelatin Inc) were added. Thereafter, at 1, 3, 5, 8, and 24 h after immersion in the

collagenase solution, three CGSs and 200 μ L of the collagenase solution from the three tubes were collected.

The concentration of HGF in the solution was measured using enzyme-linked immunosorbent assay (ELISA) kits (Human HGF immunoassay kit; R&D Systems, Inc, Minneapolis, MN) according to the manufacturer's instructions. The optical density of each solution was measured by a spectrophotometer (VersaMax; Molecular Devices, LLC, Sunnyvale, CA) at a test wavelength of 450 nm and a reference wavelength of 540 nm. The sum of the average amount of HGF released in PBS for 24 h and the average amount of HGF released in the collagenase solution for 24 h was expressed as 100%, and the amount of released HGF at each point was expressed as the percentage of the total HGF.

The collected CGSs were immediately washed with 10 mL of distilled water to stop the enzymatic reaction. The CGSs were dried using a freeze-dryer (VD-250R; Taitec Co, Ltd, Saitama, Japan) and the dry weights were measured. Then, the dried CGSs were immersed in 100 U/mL of collagenase L solution (Nitta Gelatin Inc) for 24 h to dissolve the sponges of the CGSs, and the weights of the silicon sheets were measured. The weight of the sponge of CGSs collected just after immersion in PBS was expressed as 100%, and the degraded weights (%) of the sponges of the CGSs at each time point were measured and expressed as the degradation rate.

Dual sustain and release of HGF and bFGF

Thirty-three CGSs measuring 10×15 mm in size and 3.0 mm in thickness were prepared and each CGS was initially weighed. CGSs were impregnated with both $10 \mu\text{g}/\text{cm}^2$ of HGF and $7 \mu\text{g}/\text{cm}^2$ of bFGF as follows: we prepared NSS containing the mixed solution of HGF at a concentration of $0.1 \mu\text{g}/\mu\text{L}$ and bFGF at a concentration of $0.07 \mu\text{g}/\mu\text{L}$, and applied 150 μL of this solution to the CGS. After overnight incubation at 4°C , each CGS was transferred into a 15-mL test tube and immersed in 2.5 mL of PBS. Three CGSs were immediately taken after immersion in PBS. Three CGSs and 200 μL of the solution from the three tubes were collected at 1, 3, 5, 8, and 24 h after immersion in PBS. Thereafter, CGSs were immersed in 2.5 mL of 8 U/mL collagenase L solution, and three CGSs and 200 μL of the collagenase solution from the three tubes were taken at 1, 3, 5, 8, and 24 h after immersion in the collagenase solution.

The concentration of HGF in the solution was measured as described above. The concentration of bFGF in the solution was also measured by an ELISA (Human bFGF immunoassay kit; R&D Systems, Inc) according to the manufacturer's instructions. The optical density of each solution was measured by the spectrophotometer at a test wavelength of 450 nm and a reference wavelength of 540 nm. The sum of the average amount of HGF or bFGF released in PBS for 24 h and the average amount of HGF or bFGF released in the collagenase solution for 24 h was expressed as 100%, and the amount of released HGF or bFGF at each point was expressed as the percentage of the total HGF or bFGF.

The weights of the collected CGSs were measured in the same manner as described above. The degradation rate of the sponge of CGSs was also evaluated.

Efficacy of CGSs impregnated with HGF and bFGF in murine skin defects

Preparation of CGSs impregnated with HGF or bFGF alone and both HGF and bFGF

We evaluated the healing process using CGSs impregnated with HGF or bFGF alone and both HGF and bFGF. We prepared 75 CGSs measuring 8 mm in diameter and 1.5 mm in thickness. We have set five groups: NSS group, HGF-L group (low concentration group; $10 \mu\text{g}/\text{cm}^2$ of HGF), HGF-H group (high concentration group; $50 \mu\text{g}/\text{cm}^2$ of HGF), bFGF group ($7 \mu\text{g}/\text{cm}^2$ of bFGF), and HGF + bFGF group ($10 \mu\text{g}/\text{cm}^2$ of HGF and $7 \mu\text{g}/\text{cm}^2$ of bFGF). In the NSS group, CGSs were impregnated with 50 μL of NSS. In the HGF-L group, CGSs were impregnated with 50 μL of NSS containing 5 μg of HGF. In the HGF-H group, CGSs were impregnated with 50 μL of NSS containing 25 μg of HGF. In the bFGF group, CGSs were impregnated with 50 μL of NSS containing 3.5 μg of bFGF. In the HGF + bFGF group, CGSs were impregnated with 50 μL of NSS containing both 5 μg of HGF and 3.5 μg of bFGF. The CGSs were then incubated overnight at 4°C .

Implantation of CGSs

Eight-week-old C57BL/6Jcl mice ($n = 75$, CLEA Japan Inc, Osaka, Japan) were used. Mice were anesthetized with inhalation of 5% of isoflurane (Wako Pure Chemical Industries Ltd, Osaka, Japan) during the induction period and 1.5%-2.0% of isoflurane during the maintenance period. After shaving and depilating the mice, we created a full-thickness skin defect measuring 8 mm in diameter, including the panniculus carnosus, on the back of each mouse using an 8-mm-diameter skin punch biopsy tool (Kai Industries, Gifu, Japan). After dividing the mice into five groups, we implanted each CGS on the skin defect of an animal ($n = 15$ in each group). The CGSs were sutured to the margins of the skin wound using 5-0 nylon sutures (Diadem; Wondermorks Inc, Tokyo, Japan), and sterile gauze was placed on the defect and fixed with surgical tape (Silkytex; Alcare Co, Ltd, Tokyo, Japan).

Assessment of the remaining wound area

The mice were sacrificed via the inhalation of carbon dioxide at weeks 1, 2, and 3 after CGS implantation ($n = 5$ in each group). After removing the silicone sheets of CGSs, the wounds were photographed. The remaining wound area was analyzed from the gross photographs using an imaging analyzer (ImageJ) software program, version 1.47; NIH, Bethesda, MD).

Histologic assessment of newly formed epithelium and dermis-like tissue

The specimens were harvested and sectioned axially using scalpels and scissors. Specimens were fixed with 10% formalin neutral buffer solution (Wako Pure Chemical Industries, Ltd) and paraffin embedded. Five micrometer thick sections from the center of each specimen were prepared and stained with hematoxylin and eosin and Azan staining. Microphotographs were taken using a light microscope (Biorevo BZ-9000; Keyence Corp, Osaka, Japan). The newly formed epithelium length

of each specimen was evaluated along the basal epidermal layer from the end of collagen fibers stained vivid red under the epidermis of the original wound marginal skin to the end of the newly formed epithelium on each side at 100 $\times$ magnification using an imaging software program (BZ-11analyzer, version 1.42; Keyence Corp). The average length of both sides of each specimen was calculated and used for analysis ($n = 5$).

The area of the formed dermis-like tissue was evaluated on Azan-stained sections. In the area surrounded with loose tissue above the muscle and between both levels of marginal skin, the area that was slightly lighter than the surrounding collagen fiber of the original dermis (stained dark blue) and where there was cell infiltration in the remaining sponge of the CGS was estimated using a BZ-11analyzer.

Immunohistochemical staining of newly formed capillaries and quantification of capillaries

Immunohistochemical staining of α -smooth muscle actin was performed to detect new capillaries that formed in the dermis-like tissue. We used 5-mm thick paraffin sections for staining. After deparaffinization and rehydration, antigen retrieval processing was performed using the heat-induced target retrieval method. Sections were immersed in a pre-heated staining dish containing a Tris-EDTA buffer (pH 8.0; Serva Electrophoresis GmbH, Heidelberg, Germany) and incubated for 30 min at 85°C. After being cooled to room temperature for 20 min, the sections were rinsed in Tris-buffered saline (TBS; Takara Bio Inc, Shiga, Japan) with 0.1% Tween20 (Wako Pure Chemical Industries Ltd) and then immersed in 3% hydrogen peroxide, which contains 30% hydrogen peroxide (Wako Pure Chemical Industries Ltd) and methanol (Wako Pure Chemical Industries Ltd), for 15 min to block any endogenous peroxidase activity. After being rinsed with TBS with 0.1% Tween20, Histofine Mouse Stain Kit Blocking Reagent A (Nichirei Biosciences Inc, Tokyo, Japan) was applied for 60 min at room temperature. Then, the sections were rinsed with TBS with 0.1% Tween20. The monoclonal mouse antihuman smooth muscle actin antibody (M0851; Dako Japan Co, Ltd, Tokyo, Japan) was applied as the primary antibody at a dilution of 1:500 and incubated for 30 min at room temperature, followed by washing with TBS with 0.1% Tween20 and treatment with Histofine Mouse Stain Kit Blocking Reagent B (Nichirei Biosciences Inc) for 10 min at room temperature. The sections were again rinsed with TBS with 0.1% Tween20, and the peroxidase-labeled secondary antibody, mouse anti-goat simple stain MAX PO (M) (Histofine; Nichirei Biosciences Inc), was applied for 10 min at room temperature. The sections were then rinsed with TBS with 0.1% Tween20, before being exposed to 3,3'-diaminobenzidine tetrahydrochloride (DAB; Dako Japan Co, Ltd) for 3 min at room temperature and counterstained with hematoxylin. The newly formed capillaries were counted, and the area of newly formed capillaries was measured in five 400 μ m wide square areas that were beneath the marginal skin (edge region), at the bottom of two middle regions equally divided into three sections between the marginal skin (middle region) and at the bottom of the central region of each wound (center region) in the dermis-like tissues under light microscope at 200 $\times$ magnifications. The area of newly formed capillaries was measured using a BZ-11analyzer. Only capillaries with a clearly

visible lumen were counted. The average number and area of those five areas was analyzed for statistical evaluation ($n = 5$).

Statistical analysis

Differences between the groups were examined for statistical significance using analysis of variance and the Tukey–Kramer test. All data are expressed as the mean + standard error. P values of <0.05 were considered to be statistically significant. The Microsoft Excel software program with the Statcel3 software add-in (Oms publishing, Inc, Tokyo, Japan) was used for all statistical analyses.

Results

Release of HGF and bFGF from CGS

Single release of HGF from CGS

The time course of the degradation of CGS and the release of HGF from CGS is shown in Figure 1A. During the initial 24-h period in PBS, the CGSs were not biodegraded and the amount of released HGF was $4.09 \pm 0.43 \mu\text{g}$. During the 24-h period in PBS and the 24-h period in collagenase solution, $99.3 \pm 0.5\%$ of CGSs were biodegraded and the amount of released HGF was $6.33 \pm 0.18 \mu\text{g}$. During the first 8 h in the collagenase solution, $93.1 \pm 1.5\%$ of CGSs were biodegraded and the amount of released HGF was $1.90 \pm 0.07 \mu\text{g}$. Therefore, CGSs released HGF in a sustained manner according to their biodegradation by collagenase.

Dual impregnation of HGF and bFGF and release

The time course of the degradation of CGS and the release of HGF and bFGF from CGS is shown in Figure 1B. The amount of bFGF released at 1 h after CGSs were immersed in PBS was $0.12 \pm 0.02 \mu\text{g}$. The amount of released bFGF decreased gradually in PBS. During the initial 24-h period in PBS, the CGSs were not biodegraded and the amounts of released HGF and bFGF were $1.91 \pm 0.02 \mu\text{g}$ and $0.05 \pm 0.00 \mu\text{g}$, respectively. During the 24-h period in PBS and the 24-h period in collagenase solution, $97.2 \pm 0.5\%$ of CGSs were biodegraded and the amounts of released HGF and bFGF were $4.19 \pm 0.25 \mu\text{g}$ and $0.13 \pm 0.01 \mu\text{g}$, respectively. During the first 8 h in the collagenase solution, $83.3 \pm 4.5\%$ of CGSs were biodegraded and the amounts of released HGF and bFGF were $1.25 \pm 0.09 \mu\text{g}$ and $0.08 \pm 0.01 \mu\text{g}$, respectively. Therefore, CGSs released HGF and bFGF simultaneously in a sustained manner according to their biodegradation by collagenase.

Efficacy of CGS impregnated with HGF or bFGF in murine skin defects

Assessment of the remaining wound area

The gross appearances of wounds after the implantation of CGSs are shown in Figure 2A. The time course of the remaining wound area is shown in Figure 2B. The remaining wound area of the wound of the HGF-L group was significantly smaller than that of the NSS group at week 1 ($P < 0.01$). At week 2, the remaining wound areas of the wounds of the bFGF and HGF + bFGF groups were significantly smaller than those

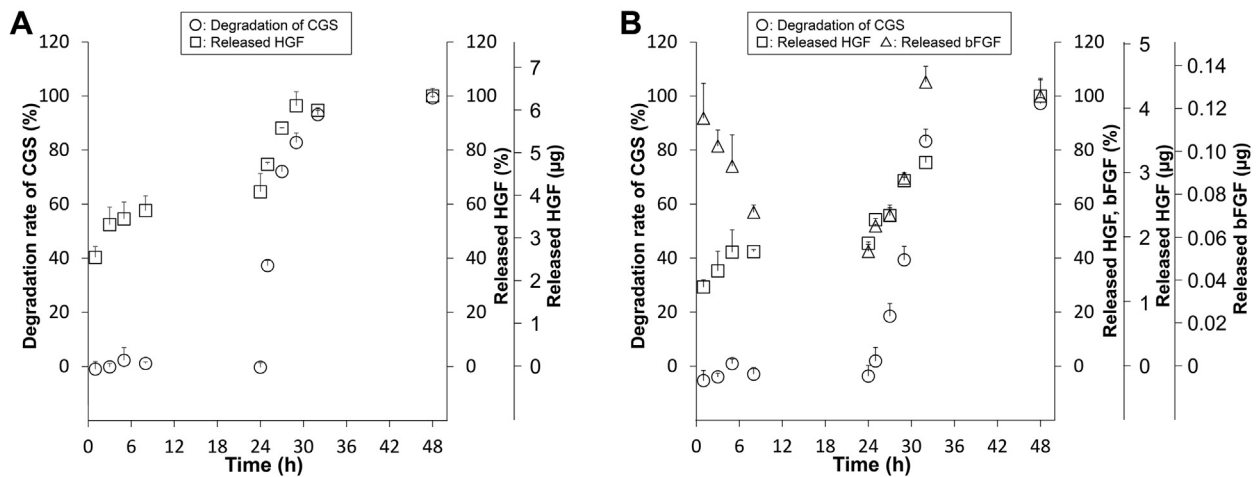


Fig. 1 – The degradation of CGS and the release of growth factors. (A) The time course of the degradation of CGS and the release of HGF. The solution was changed from PBS to a collagenase solution at 24 h. CGS was not degraded in PBS. CGS began to be degraded in the collagenase solution and HGF was subsequently released. Data are presented as the mean + standard error. (B) The time course of the degradation rate of CGS and the release of HGF and bFGF. The solution was changed from PBS to a collagenase solution at 24 h. CGS was not degraded in PBS. CGS began to be degraded in the collagenase solution and both HGF and bFGF were subsequently released. Data are presented as the mean + standard error.

of the NSS and HGF-H groups (bFGF group $P < 0.01$ versus NSS group, $P < 0.05$ versus HGF-H group; HGF + bFGF group $P < 0.01$ versus NSS group, $P < 0.05$ versus HGF-H group). At week 3, the remaining wound area of the wound of the NSS group was significantly larger than those of the HGF-L, HGF-H and bFGF groups ($P < 0.01$ versus HGF-H group, $P < 0.05$ versus HGF-L and bFGF groups).

Histologic assessment of the neoepithelium length

Micrographs of hematoxylin and eosin–stained sections are shown in Figure 3A. The time course of the neoepithelium length is shown in Figure 3B. The neoepithelium length at week 3 was not assessed because most of the wounds at week 3 have been completely epithelized. At week 1, the length of the neoepithelium in the HGF-L group was significantly longer than that of the NSS group ($P < 0.05$). At week 2, the length of the neoepithelium in the bFGF group was significantly longer than that of the NSS group ($P < 0.05$). The length of the neoepithelium in the HGF + bFGF group was the longest and significantly longer than those in the NSS and HGF-H groups ($P < 0.01$ for each).

Histologic assessment of the dermis-like tissue

Micrographs of Azan-stained sections are shown in Figure 4A. The histological assessment of dermis-like tissue has not been performed at week 1 because it was difficult to distinguish dermis-like tissue from implanted CGS. The formed dermis-like tissues in the NSS group at weeks 2 and 3 and that of the HGF-H group at week 2 were thin. The formed dermis-like tissues in the bFGF and HGF + bFGF groups at weeks 2 and 3 were thick. The formed dermis-like tissue in the HGF-L group at week 3 was thick. The time course of the area of the dermis-like tissue is shown in Figure 4B. The formed dermis-like tissues in the bFGF and HGF + bFGF groups were larger than those of the NSS, HGF-L, and HGF-H groups at week 2 (bFGF group $P < 0.05$ versus HGF-L group and $P < 0.01$ versus NSS and HGF-H groups;

HGF + bFGF group $P < 0.01$ versus NSS, HGF-L and HGF-H groups). The formed dermis-like tissue in the HGF-L group was larger than that of the NSS group at week 2 ($P < 0.05$). The formed dermis-like tissues in the bFGF and HGF + bFGF groups were larger than that of the NSS group at week 3 ($P < 0.05$).

Evaluation of newly formed capillaries

Micrographs of anti- α -smooth muscle actin staining are shown in Figure 5A. Many capillaries were observed in the dermis-like tissues of the bFGF and HGF + bFGF groups in the central and middle regions. A quantitative analysis of neovascularization is indicated in Figure 5B and C. The number of newly formed capillaries in the bFGF and HGF + bFGF groups were greater than those in the NSS and HGF-H groups (all $P < 0.01$). The number of newly formed capillaries in the HGF + bFGF group was greater than those in the HGF-L and bFGF groups ($P < 0.01$ and $P < 0.05$, respectively). The area of newly formed capillaries in the HGF + bFGF group was larger than those in the NSS and HGF-H groups ($P < 0.05$ and $P < 0.01$, respectively).

Discussion

In this study, we indicated that our CGSs could sustain HGF as well as bFGF; in addition, CGSs could sustain HGF and bFGF simultaneously. Sustained HGF or both HGF and bFGF were released from CGSs according to their biodegradation.

In our release test, about 60% of HGF was released in the PBS. It has been reported that HGF, which is positively charged, ionically interacts with acidic gelatin, and HGF, after once interacting with acidic gelatin, cannot be released unless gelatin degradation takes place.²⁶ Therefore, during the immersion of CGSs in PBS, HGF without ionically connected gelatin molecules was released as the initial burst through simple diffusion. HGF released in the collagenase solution occurred through the enzymatic degradation of CGS.

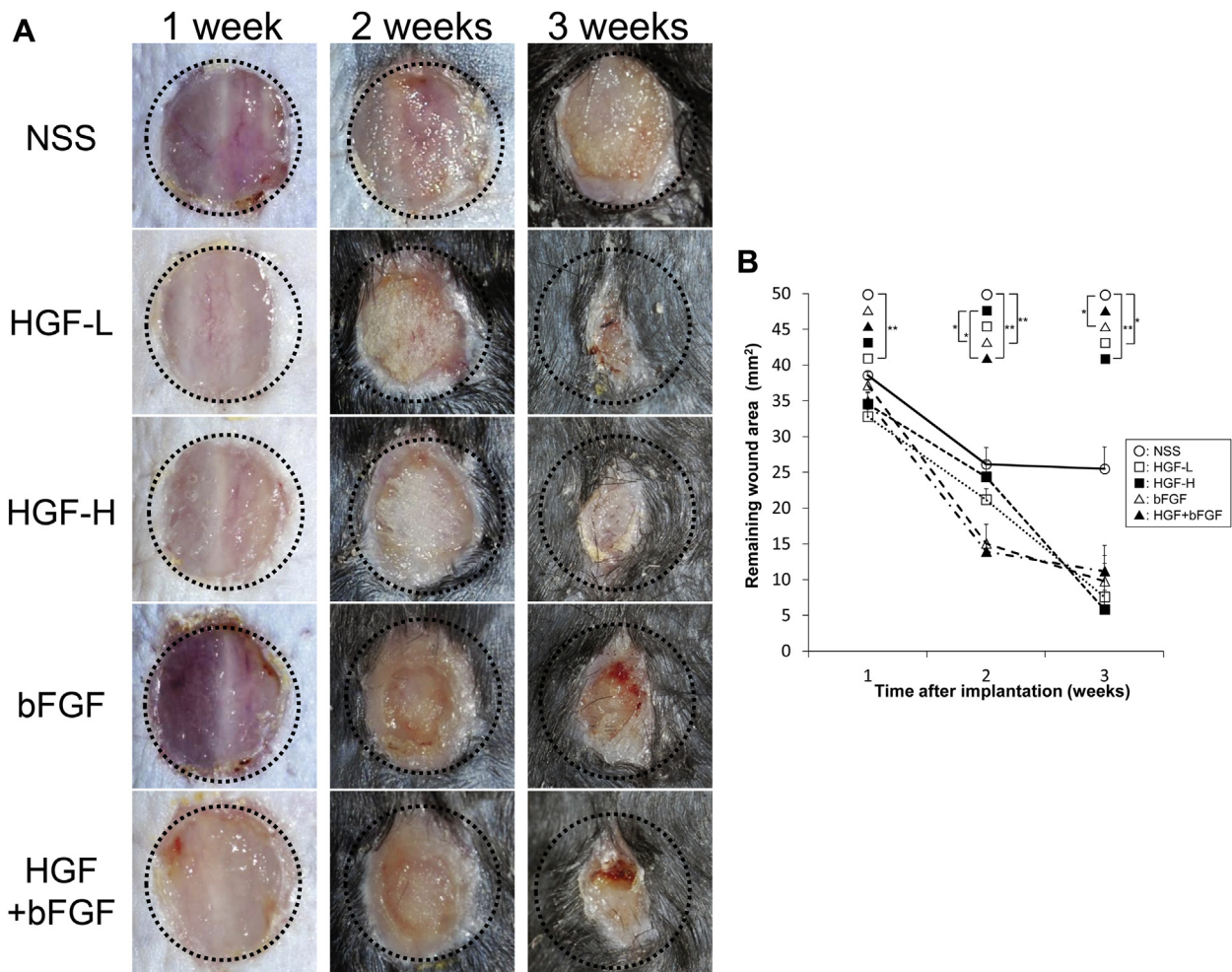


Fig. 2 – The time course of wound closure. (A) The gross appearances of the wounds. The wounds in the NSS group remained even at week 3. On the other hand, the wound areas of the other four groups were decreased at week 3. The black circles indicate the original wound area (8 mm in diameter). **(B)** The time course of the remaining wound area. The remaining wound area of the HGF-L group was smaller than that of the NSS group at week 1 ($P < 0.01$). At week 2, the remaining wound areas of the bFGF and HGF + bFGF groups were smaller than those of the NSS and HGF-H groups (bFGF group $P < 0.01$ versus NSS group, $P < 0.05$ versus HGF-H group; HGF + bFGF group $P < 0.01$ versus NSS group, $P < 0.05$ versus HGF-H group). At week 3, the wound area of the NSS group was larger in the remaining wound area than those of the HGF-L, HGF-H, and bFGF groups ($P < 0.01$ versus HGF-H group, $P < 0.05$ versus HGF-L and bFGF groups). Data are presented as the mean + standard error. * $P < 0.05$, ** $P < 0.01$. (Color version of figure is available online.)

Regarding the bFGF released in the dual release test of HGF and bFGF, the total amount of released bFGF decreased in PBS over 24 h, whereas it increased in collagenase solution. In previous reports, we showed that the amount of bFGF gradually decreased during incubation in polypropylene tubes at 37°C.²⁷ This is mainly because of the absorption of bFGF molecules to the wall of the polypropylene tube, and the aggregation or the denaturation of bFGF could be minor reasons. Our result showed that the amount of bFGF increased again after the degradation of CGS in collagenase solution, therefore, the simultaneous release of bFGF and HGF was confirmed in our results.

Wound closure is achieved by wound contraction and epithelization. Wound contraction causes severe clinical problems, such as a limitation in the joint range of motion or hypertrophic scarring in the human wound healing process. In this study, the HGF-L group showed superior

epithelization compared with the HGF-H group at week 1. This indicates that HGF at 10 $\mu\text{g}/\text{cm}^2$ is appropriate for epithelization compared with a higher dosage of 50 $\mu\text{g}/\text{cm}^2$. HGF-L also promoted dermis-like tissue formation at week 2; however, the bFGF group promoted capillary formation and dermis-like tissue formation superior to the HGF-L group at week 2. As we have shown in our previous study, bFGF has superior angiogenic potency and thus accelerates dermis-like tissue formation in combination with CGS. Our result showed that HGF is inferior to bFGF in angiogenesis and dermis-like tissue formation, although superior in epithelization, especially within 2 weeks after administration. In the wound healing process of humans, the regeneration of dermis-like tissue is important to prevent wound contraction, and we have shown that the superior formation of dermis-like tissue by bFGF prevented wound contraction.²⁸

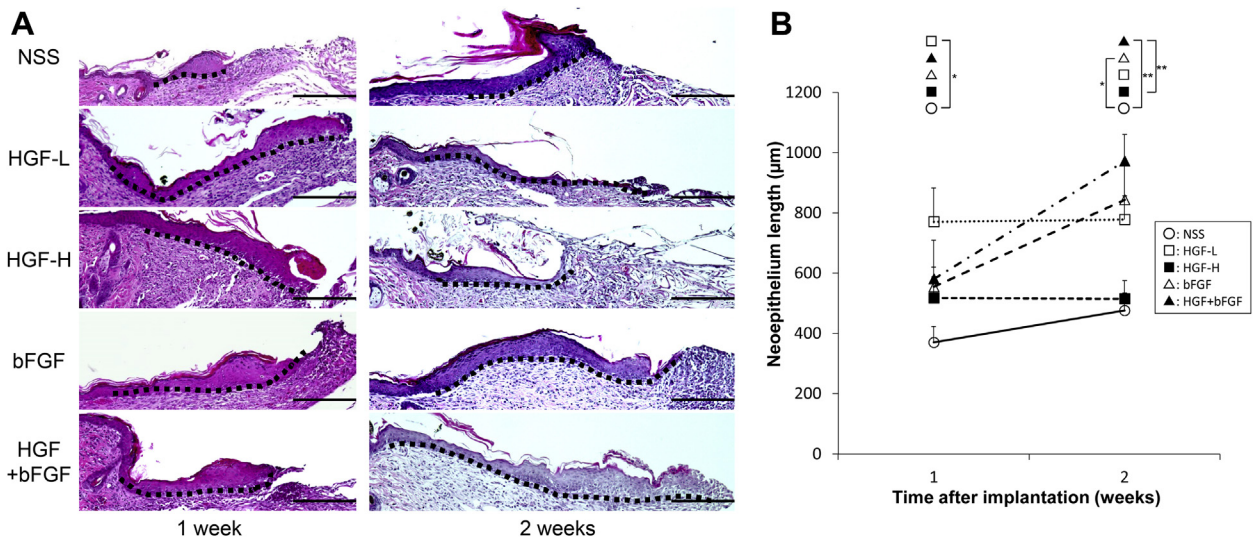


Fig. 3 – Assessment of the neoepithelium length. (A) Microphotographs of HE sections at weeks 1 and 2. The neoepithelium is indicated by a broken line. The neoepithelium of the HGF-L group at week 1 was long and the neoepithelium of the bFGF and HGF + bFGF groups at week 2 were long. Bar: 200 μm . **(B)** The time course of the neoepithelium length. The length of the neoepithelium of the HGF-L group was significantly longer than that of the NSS group at week 1 ($P < 0.05$). The length of the neoepithelium of the bFGF group was significantly longer than that of the NSS group at week 2 ($P < 0.05$). The length of the neoepithelium of the HGF + bFGF group was significantly longer than those of the NSS and HGF-H groups at week 2 ($P < 0.01$). Data are presented as the mean + standard error. * $P < 0.05$, ** $P < 0.01$. (Color version of figure is available online.)

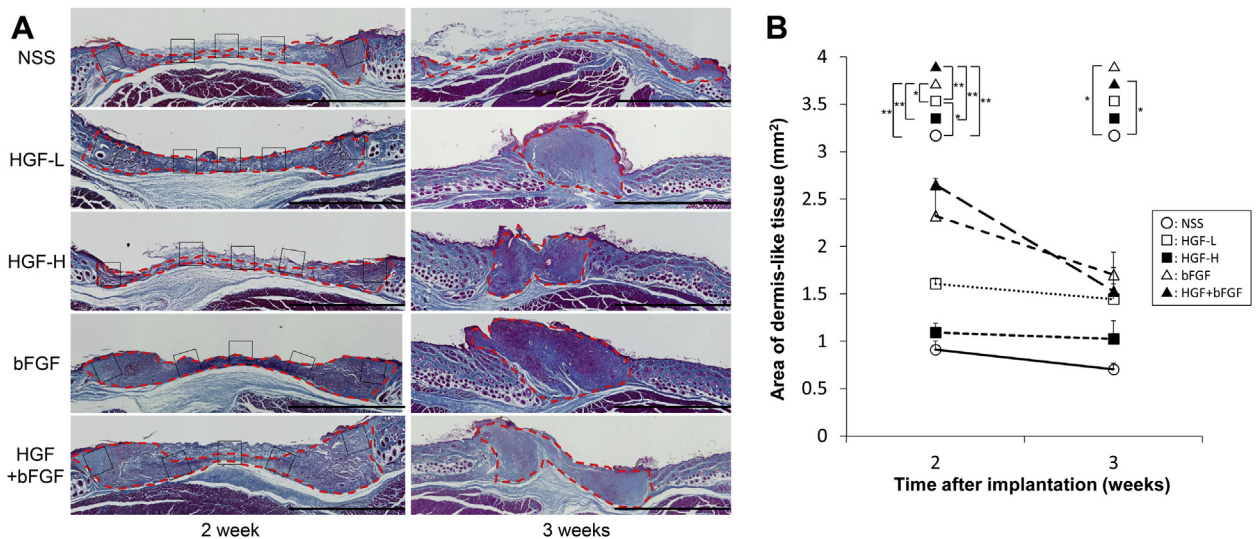


Fig. 4 – Assessment of dermis-like tissue. (A) Azan-stained sections of wounds at weeks 2 and 3. The area that was slightly lighter than the surrounding collagen fiber of the original dermis (stained dark blue) and where there was cell infiltration in the remaining sponge of the CGS was estimated using a BZ-llanalyzer. Broken lines indicate the areas of dermis-like tissue. The solid squares (400 μm in width and height) show the area used for the evaluation of newly formed capillaries by immunohistochemical staining in Figure 5A. The area was beneath both the marginal skin (edge region), at the bottom of two central regions equally divided into three parts between the marginal skin (middle region) and at the bottom of the central region of each wound (central region) in the dermis-like tissues. Bar: 2 mm. **(B)** The time course of the area of dermis-like tissue. At weeks 2 and 3, the newly formed dermis-like tissues of wounds of the bFGF and HGF + bFGF groups were larger at week 2 compared with those of the NSS, HGF-L, and HGF-H groups (bFGF group $P < 0.05$ versus HGF-L group and $P < 0.01$ versus NSS and HGF-H groups; HGF + bFGF group $P < 0.01$ versus NSS, HGF-L and HGF-H groups). The newly formed dermis-like tissue of wounds of the HGF-L group was larger at week 2 compared with that of the NSS group ($P < 0.05$). The newly formed dermis-like tissues of wounds of the bFGF and HGF + bFGF groups were larger at week 3 compared with that of the NSS group ($P < 0.05$). Data are presented as the mean + standard error. * $P < 0.05$, ** $P < 0.01$. (Color version of figure is available online.)

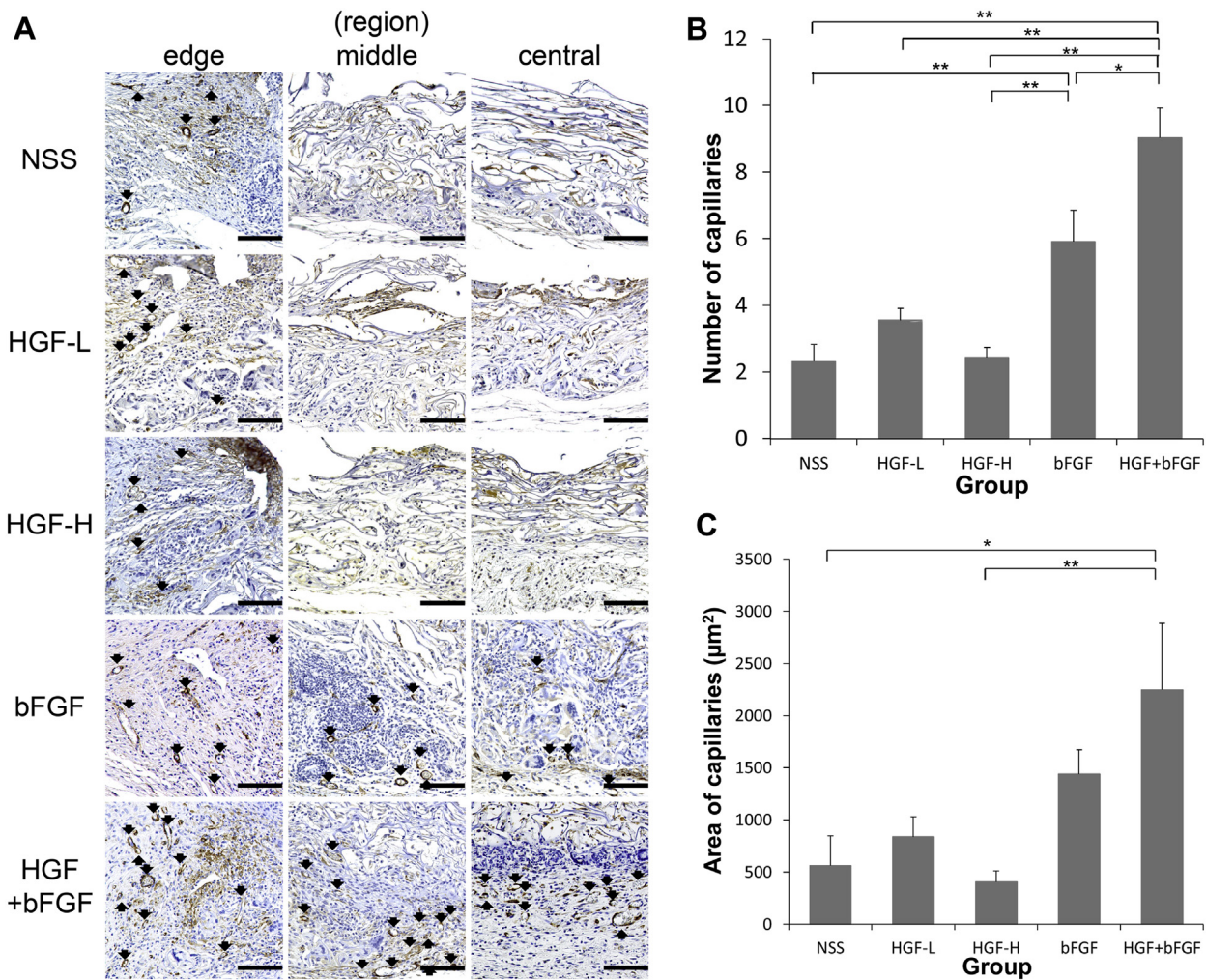


Fig. 5 – Comparison of newly formed capillaries in each dermis-like tissue. (A) Newly formed capillaries in the dermis-like tissues immunostained with α SMA at week 2. Abundant capillaries were observed in all groups at the edge region and in bFGF and HGF + bFGF groups at the central and middle regions. Black arrows indicate capillaries. Bar: 100 μ m. **(B)** Comparison of the number of newly formed capillaries at week 2. The number of newly formed capillaries in the HGF + bFGF group was greater than those in the other four groups ($P < 0.01$ versus NSS, HGF-L, and HGF-H, $P < 0.05$ versus bFGF). The number of newly formed capillaries in the bFGF group was greater than those in the NSS and HGF-H groups ($P < 0.01$). Data are presented as the mean + standard error. * $P < 0.05$, ** $P < 0.01$. **(C)** Comparison of the area of newly formed capillaries at week 2. The area of newly formed capillaries in the HGF + bFGF group was larger than those in the NSS and HGF-H groups ($P < 0.01$ versus HGF-H, $P < 0.05$ versus NSS). Data are presented as the mean + standard error. * $P < 0.05$, ** $P < 0.01$. (Color version of figure is available online.)

However, epithelization is generally not promoted in the earlier stage (within 1 week) after the application of bFGF, and epithelization will be observed after the adequate formation of dermis-like tissue several weeks after bFGF application. In this study, the HGF + bFGF group showed the best results in epithelization, wound closure, capillary formation, and dermis-like tissue formation. Therefore, this combination therapy of CGS with bFGF and HGF is superior to bFGF treatment in earlier epithelization. Concerning the inflammatory process and fibrosis that are important factors during the wound healing process, apparent inflammation that prolonged the wound healing was not observed and fibrosis was not observed histologically in the observation period. In the next step, we are willing to evaluate protein

levels or gene expression and quantitatively detect inflammation and fibrosis.

Regarding the dual release of growth factors, it was initially reported that vascular endothelial growth factor and PDGF released from a single, structural polymer scaffold resulted in rapid vascular formation, indicating the importance of the action of multiple growth factors in tissue regeneration and engineering.²⁹ Another report about the dual release of HGF and bFGF showed that the sustained dual release of bFGF and HGF from a collagen microsphere enhanced blood vessel formation compared with bFGF or HGF released alone.³⁰ In this study, there was a significant difference in the newly formed capillary formation between the bFGF group and the HGF + bFGF group. We have previously reported that superior angiogenesis by the

sustained release of growth factors such as bFGF or PDGF from CGS-accelerated dermis-like tissue formation *in vivo*.^{12,24} Angiogenesis is an important factor for dermis-like tissue formation. The dual release of HGF and bFGF from CGS is a promising treatment option for full-thickness skin defects.

We focused on HGF as the growth factor to be impregnated into CGS because HGF is positively charged and a promising growth factor for future clinical use. Regarding the potential use of CGS, we have shown the release of some positively charged growth factors from the CGS. We can arrange the various growth factors including negatively charged growth factors according to the IEP of gelatin. Therefore, further investigations on multiple growth factors in combination with CGS are also necessary to improve the wound healing process.

Conclusions

Our findings indicated that CGS had the ability to sustain and release HGF alone or HGF and bFGF simultaneously. CGS impregnated with HGF promoted epithelialization. CGS impregnated with HGF in addition to bFGF promoted angiogenesis, and the dual sustained release of HGF and bFGF from CGS was a promising treatment option for full-thickness skin defects.

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Author's contributions: S.O. and N.M. conceived and designed the experiments. S.O., N.M., M.S., and C.J. performed the experiments. S.O., N.M., and S.S. analyzed the data. Y.S. and T.T. prepared materials. S.O. and N.M. wrote the paper.

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Disclosure

The authors report no proprietary or commercial interest in any of the products mentioned or any of the concepts discussed in this article.

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