



Original Article

Heparin-conjugated collagen as a potent growth factor-localizing and stabilizing scaffold for regenerative medicine

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ABSTRACT

Introduction: Growth factors are crucial bioactive molecules in vitro and in vivo. Among them, basic fibroblast growth factor (bFGF) has been used widely for various applications such as cell culture and regenerative medicine. However, bFGF has extremely poor stability in aqueous solution; thus, it is difficult to maintain its high local concentration. Heparin-conjugated materials have been studied recently as promising scaffold-immobilizing growth factors for biological and medical applications. The previous studies have focused on the local concentration maintenance and sustained release of the growth factors from the scaffold.

Methods: In this paper, we focused on the biological stability of bFGF immobilized on the heparin-conjugated collagen (hep-col) scaffold. The stability of the immobilized bFGF was quantitatively evaluated at physiological temperature (37 °C) using cell culture and ELISA.

Results: The immobilized bFGF had twice higher stability than the bFGF solution. Furthermore, the hep-col scaffold was able to immobilize not only bFGF but also other growth factors (i.e., vascular endothelial growth factor and hepatocyte growth factor) at high efficiency.

Conclusions: The hep-col scaffold can localize several kinds of growth factors as well as stabilize bFGF under physiological temperature and is a promising potent scaffold for regenerative medicine.

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1. Introduction

Growth factors are biomolecules that effectively regulate the specific functions and growth activity of cells constituting tissues in vitro and in vivo. Therefore, they have been used recently for a wide range of applications such as cell culture and regenerative

medicine. For instance, basic fibroblast growth factor (bFGF) is essential for maintaining the undifferentiated state of human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) [1–3] and contributes to the stable supply of these cells for various uses. In addition, bFGF induces angiogenesis and enables the delivery of in vivo-secreted bioactive molecules to lesion sites, thereby promoting tissue regeneration [4]. However, bFGF has poor stability and is expensive [5,6]. Moreover, when bFGF is administered in vivo in an aqueous solution, it is difficult to maintain a high local concentration since bFGF diffuses from the injection site. Hence, at present, it is necessary to reduce the costs due to high doses of bFGF used to obtain the desired efficacy such as the long-term stem cell maintenance and the angiogenic therapy.

Recently, immobilization onto heparin-conjugated scaffolds has been used to immobilize growth factors [7–10]. Heparin is a sulfated glycosaminoglycan, the representative component of the extracellular matrix, and has an electrostatic affinity to various kinds of growth factors including bFGF [11,12]. In addition, heparin stabilizes growth factors by complexation [13,14], and thus is added to the culture medium as a growth factor-stabilizing agent.

Abbreviations: bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; C-FBS, charcoal/dextran-treated fetal bovine serum; CMF-PBS, calcium- and magnesium-free phosphate-buffered saline; EDC, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; hep-col, heparin-conjugated collagen; hESCs, human embryonic stem cells; HGF, hepatocyte growth factor; hiPSCs, induced pluripotent stem cells; HUVECs, human umbilical vein vascular endothelial cells; MES, 2-morpholinoethanesulfonic acid; monohydrate, NHS; N-hydroxysuccinimide, VEGF; vascular endothelial growth factor.

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Therefore, heparin-conjugated scaffolds allow spontaneous immobilization, in vivo localization, and half-life prolongation of growth factors without chemical crosslinking, which causes their denaturation.

Previous studies have demonstrated high efficiency of growth factor immobilization using heparin-conjugated collagen (hep-col) [7,15,16]. However, these studies have mainly focused on localization and sustained release of growth factors, but few have considered their stability. Moreover, the stability of growth factors immobilized onto the hep-col scaffold has been evaluated only at 4 °C [7], which is a non-physiological condition.

The stability of vascular endothelial growth factor (VEGF) immobilized on hep-col films was evaluated in our previous study [17]. We assessed cell proliferation as a readout for the growth behavior of human umbilical vein vascular endothelial cells (HUVECs) on the VEGF immobilized on hep-col films using ELISA and cell culturing. We quantified the amount of VEGF that retained bioactivity during the pre-incubation period at 37 °C and concluded that immobilization on hep-col films improved the stability of VEGF.

In this study, we explored the potential of hep-col films as scaffolds for regenerative medicine. We immobilized bFGF, which has particularly poor stability, on the hep-col film, and quantitatively evaluated the stability under physiological conditions using HUVECs. We also evaluated the ability of hep-col scaffolds to immobilize different types of growth factors (e.g., bFGF, VEGF, and hepatocyte growth factor [HGF]).

2. Materials and methods

2.1. Cell and culture media

HUVECs and the HuMedia-EG2 culture medium were purchased from Kurabo (Osaka, Japan). The HuMedia-EG2 medium contains the basal medium HuMedia-EB2, 2% (v/v) fetal bovine serum (FBS), 1 µg/mL hydrocortisone, 50 µg/mL gentamicin, 50 ng/mL amphotericin B, 10 ng/mL human recombinant epidermal growth factor (EGF), 5 ng/mL human recombinant basic fibroblast growth factor (bFGF), and 10 µg/mL heparin. C-FBS/HuMedia-EG2 is a modification of the HuMedia-EG2 culture medium and contains only 2% (v/v) charcoal/dextran-treated FBS (C-FBS, Thermo Fisher Scientific, Kanagawa, Japan), 1 µg/mL hydrocortisone, 50 µg/mL gentamicin, and 50 ng/mL amphotericin B in the HuMedia-EB2.

2.2. Fabrication of heparin-conjugated collagen film and evaluation of growth factor immobilizability

The heparin-conjugated collagen film was prepared as previously reported [17]. In short, Collagen Type I–C (3 mg/mL; pH 3.0; Nitta Gelatin, Osaka, Japan) was diluted to 0.3 mg/mL by adding HCl (pH 3.0). Two hundred and fifty microliters of the collagen solution were dispensed into each well of a 48-well microplate ($A = 1 \text{ cm}^2$), followed by air-drying on a clean bench for 1 day to obtain the film. A 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS) solution was prepared by adding 8 mg/mL EDC (Peptide Institute Inc., Osaka, Japan) and 2 mg/mL NHS (Fujifilm, Wako Pure Chemical Industries, Osaka, Japan) to 0.05 M 2-morpholinoethanesulfonic acid, monohydrate (MES, pH 5.6, Dojindo Laboratories, Kumamoto, Japan) buffer. The collagen films were washed with the MES buffer and were then crosslinked by immersing into 200 µL of the EDC/NHS solution for 4 h.

Subsequently, a heparin solution was prepared by adding 1 mg/mL heparin sodium (Fujifilm, Wako Pure Chemical Industries, Osaka, Japan), 1 mg/mL EDC, and 0.6 mg/mL NHS to 1 mL of 0.05 M MES buffer. To conjugate heparin to collagen, 200 µL of the heparin

solution was poured over the collagen films, washed with the MES buffer, and allowed to incubate for 2 h at room temperature (RT).

Human bFGF and the ELISA kit (DuoSet ELISA Development Systems) were purchased from R&D Systems Inc (Minneapolis, MN, USA). One percent (w/v) bovine serum albumin (BSA, Fujifilm, Wako Pure Chemical Industries, Osaka, Japan) solution was prepared in calcium- and magnesium-free phosphate-buffered saline (CMF-PBS). To suppress unspecific adsorption of bFGF, the heparin-conjugated collagen films (hep-col film; about 20 µg/cm² heparin) were blocked with 500 µL of the BSA solution for 1 h at RT following CMF-PBS washing. Solutions containing bFGF at various concentrations (0–100 ng/mL) were prepared in the BSA solution and kept on ice until use. Two hundred microliters of each bFGF solution were added individually to the hep-col films and incubated for 3 h at RT to immobilize bFGF.

Unbound bFGF concentration in the supernatant of each film was evaluated using the DuoSet ELISA Development Systems kit. bFGF solutions for calculating the calibration curve were prepared from the bFGF solutions added to the hep-col films. The immobilized bFGF density on the film was estimated by subtracting the unbound bFGF concentration from the total bFGF concentration. The ratio of immobilized bFGF was calculated by dividing the amount of immobilized bFGF by that of the initial. For rat VEGF (R&D Systems, Minneapolis, MN, USA) and mouse HGF (R&D Systems, Minneapolis, MN, USA), immobilization and quantification of growth factors were performed in the same manner as for bFGF.

2.3. Cell culture of human umbilical vein vascular endothelial cells

For cell culture, we used blocking treatment with C-FBS to suppress nonspecific adsorption of bFGF. A 10% (v/v) C-FBS solution was prepared by diluting with CMF-PBS. The hep-col films (about 20 µg/cm² heparin) were then blocked with 500 µL of the C-FBS solution for 1 h at RT. Solutions containing bFGF at various concentrations (0–50 ng/mL) were prepared in C-FBS/HuMedia-EG2 medium. To immobilize bFGF, 200 µL of each bFGF solution was added separately to the hep-col films and incubated for 3 h at RT following PBS washing. After immobilization, the supernatants were collected, and the unbound bFGF concentrations were measured as described above.

HUVECs were then seeded on the films at a density of 5×10^3 cells/cm² growth factor/heparin-free Humedia-EG2 medium and cultured under standard conditions (37 °C; 5% CO₂; 95% air). After 1 day, the culture medium was replaced with the C-FBS/HuMedia-EG2 and every 2 days afterward.

Cells contain a certain amount of dehydrogenase. When the cell morphology does not change significantly, the intracellular dehydrogenase activity increases proportionally with the number of viable cells. As an index of viable cell number, intracellular dehydrogenase activity of HUVECs was evaluated using the WST-8 assay (Cell Counting Kit-8, Dojindo, Japan). The WST-8 solution was mixed with the HuMedia-EG2 medium at a volume ratio of 1:10. The culture medium with HUVECs was replaced with the WST-8 medium mixture and incubated for 4 h under standard culture conditions. After the incubation, 0.1 N HCl at a volume equal to that of the WST-8 was added to stop the reaction. The supernatant was transferred to a 96-well plate, and its absorbance at 450 nm was measured using a microplate auto colorimeter (Immuno Mini NJ-2300, Biotec Co., Tokyo, Japan).

2.4. Stability of basic fibroblast growth factor

To evaluate the stability of the immobilized bFGF, 50 ng/mL of bFGF solution was prepared in the C-FBS/HuMedia-EG2 medium. After blocking with the C-FBS solution, 200 µL of each bFGF solution

was added to the hep-col films and kept for 3 h at RT. The supernatant was replaced with the C-FBS/HuMedia-EG2 medium, and the hep-col films with the immobilized bFGF were then pre-incubated under cell culture conditions for 7 days.

To evaluate the stability of bFGF in culture medium, the bFGF solution was pre-incubated under cell culture conditions for 7 days. After blocking with the C-FBS solution, 200 μ L of the pre-incubated bFGF solution was added to hep-col films and then left for 3 h at RT.

HUVECs were placed onto two kinds of hep-col films with the immobilized pre-incubated bFGF at a density of 5×10^3 cells/cm² using the growth factor/heparin-free HuMedia-EG2 medium and grow on the C-FBS/HuMedia-EG2 medium. The culture medium was replaced with the WST-8 medium mixture after 1, 3, or 5 days, and the intracellular dehydrogenase activity of HUVECs was evaluated by the WST-8 assay.

2.5. Statistical analysis

The results were expressed as mean \pm standard deviation. Statistical analysis was performed using a two-tailed unpaired Student's t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Growth factor immobilizability on heparin-conjugated collagen films

The results of ELISA indicated that the density of the immobilized bFGF, VEGF, and HGF increased almost linearly with their initial concentration (Fig. 1A and B, and C). The efficiency of growth factor immobilization via hep-col films was about 80–90% in the

range from 0 to 100 ng/mL, respectively, and these concentrations are several-fold higher than that of cell culture medium. Moreover, the immobilizing efficiency of growth factors on the hep-col films was always higher than that of a heparin-free collagen film. Therefore, the hep-col film can spontaneously immobilize several kinds of growth factors at a high efficiency.

3.2. Dependence of cell growth behavior on the amount of immobilized growth factor

The intracellular dehydrogenase activity of HUVECs cultured on the hep-col film-immobilized bFGF was higher than that under the bFGF-free condition and increased more rapidly during the 5-day culture period (Fig. 2 A). In addition, the intracellular dehydrogenase activity of HUVECs cultured on the films increased with an increase in the immobilized bFGF amount. Furthermore, when the intracellular dehydrogenase activity of HUVECs was plotted against the bFGF immobilized density on the 5th day in culture, a positive correlation was found (Fig. 2 B). Light microscopic observations confirmed no obvious differences in cell morphology either with bFGF immobilization or with the culture period, indicating that the increase in intracellular dehydrogenase activity was due to cell proliferation (Fig. 2 C, D, E, and F). Therefore, the amount of active bFGF can be estimated from the intracellular dehydrogenase activity of HUVECs on the 5th day in culture.

3.3. Growth factor biological stability at physiological temperature

Hep-col films with the immobilized bFGF at a density of 9.87 ng/cm² were pre-incubated for various periods (0–7 days) at 37 °C. Intracellular dehydrogenase activity of HUVECs cultured on the

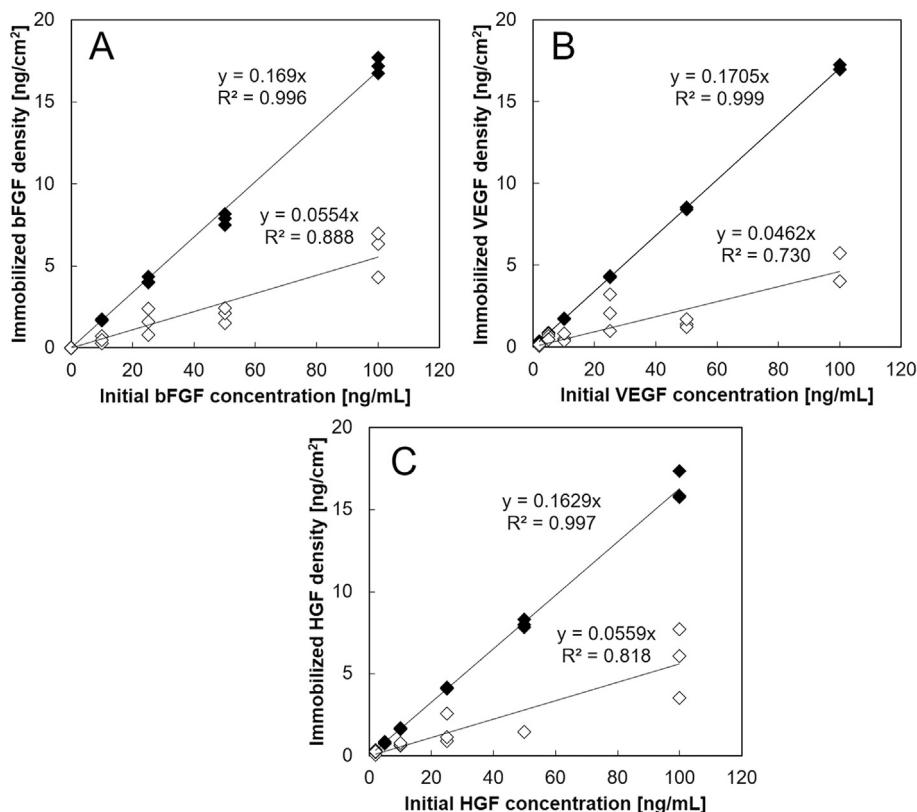


Fig. 1. Growth factor immobilizability on heparin-conjugated collagen film. Immobilized density of bFGF (A), VEGF (B), and HGF (C) at various concentrations (closed diamonds, heparin-conjugated collagen; open diamonds, collagen film; n = 3).

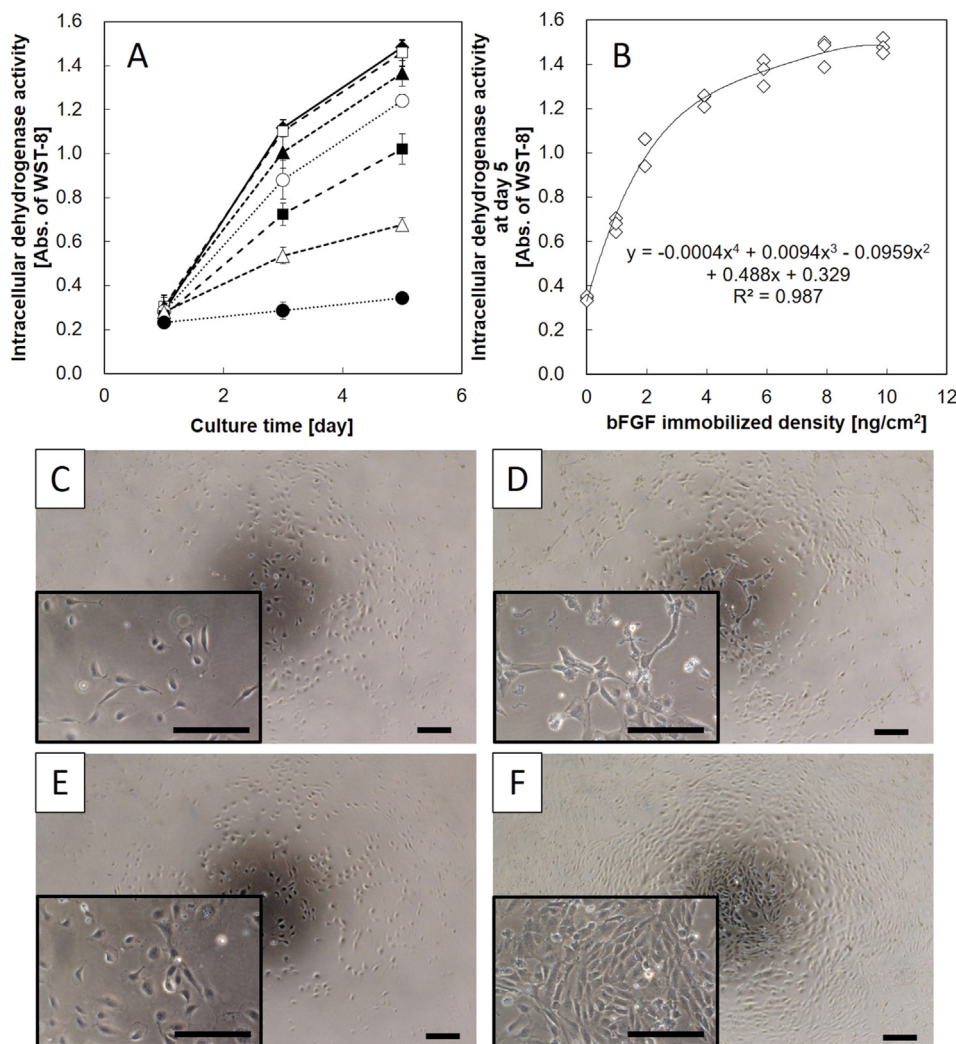


Fig. 2. Intracellular dehydrogenase activity and morphology of HUVECs cultured on hep-col films at various immobilized bFGF densities (A) Changes in intracellular dehydrogenase activity over time (closed diamonds, 9.87 ng/cm²; open squares, 7.91 ng/cm²; closed triangles, 5.89 ng/cm²; open circles, 3.93 ng/cm²; closed squares, 1.95 ng/cm²; open triangles, 0.97 ng/cm²; closed circles, 0 ng/cm²; n = 3). Bars represent SD (B) Calibration curve between immobilized bFGF density and intracellular dehydrogenase activity after 5 days in culture (C–D) Morphologies of HUVECs cultured on hep-col film immobilized with 0 ng/cm² bFGF after 1 day in culture (C) and 5 days in culture (D) (E–F) Morphologies of HUVECs cultured on hep-col film immobilized with 9.87 ng/cm² bFGF after 1 day in culture (E) and 5 days in culture (F). Bars indicate 200 μm.

pre-incubated films increased during the 5-day culture period (Fig. 3 A). However, the increase in the intracellular dehydrogenase activity over the culture period slowed gradually with prolongation of the pre-incubation period of the films.

Similarly, 50 ng/mL of the bFGF solution was pre-incubated at 37 °C (0–7 days), followed by bFGF immobilization on hep-col films. When HUVECs were cultured on these films, the intracellular dehydrogenase activity increased over the 5-day culture period (Fig. 3 B) in a similar manner as when bFGF was immobilized before pre-incubation (Fig. 3 A). The intracellular dehydrogenase activity of HUVECs hardly increased when the bFGF solution was pre-incubated prior to immobilization, unlike the condition when bFGF-immobilized films were pre-incubated for the same period. These results implied that both the immobilized bFGF and bFGF in the solution were deactivated by pre-incubation at 37 °C. Nonetheless, the intracellular dehydrogenase activity was always higher when bFGF was immobilized during the pre-incubation period (Fig. 3C).

Furthermore, we used the calibration curve showing the correlation between HUVEC intracellular dehydrogenase activity on

the 5th day in culture and density of immobilized bFGF (Fig. 2 B), to calculate the amount of bFGF with retained biological activity in each culture system (Fig. 3 D). When the bFGF solution was pre-incubated at 37 °C for 3 days or more, almost all bFGF lost biological activity, whereas approximately 60% of bFGF immobilized on the hep-col maintained biological activity even after 3 days pre-incubation. Moreover, the hep-col-immobilized bFGF was better at retaining biological activity than bFGF in the solution after 7 days pre-incubation, implying that heparin-mediated immobilization improved the biological stability of bFGF.

4. Discussion

Growth factors are signal molecules used in a wide range of applications such as cell culture and regenerative medicine. Among them, bFGF has been used for various purposes such as cell culture, in vivo nerve regeneration, wound healing, and inducing angiogenesis. However, since the stability of bFGF is poor, an excess amount of bFGF is used to obtain a certain efficacy and the resulting increase in the costs pose a significant problem.

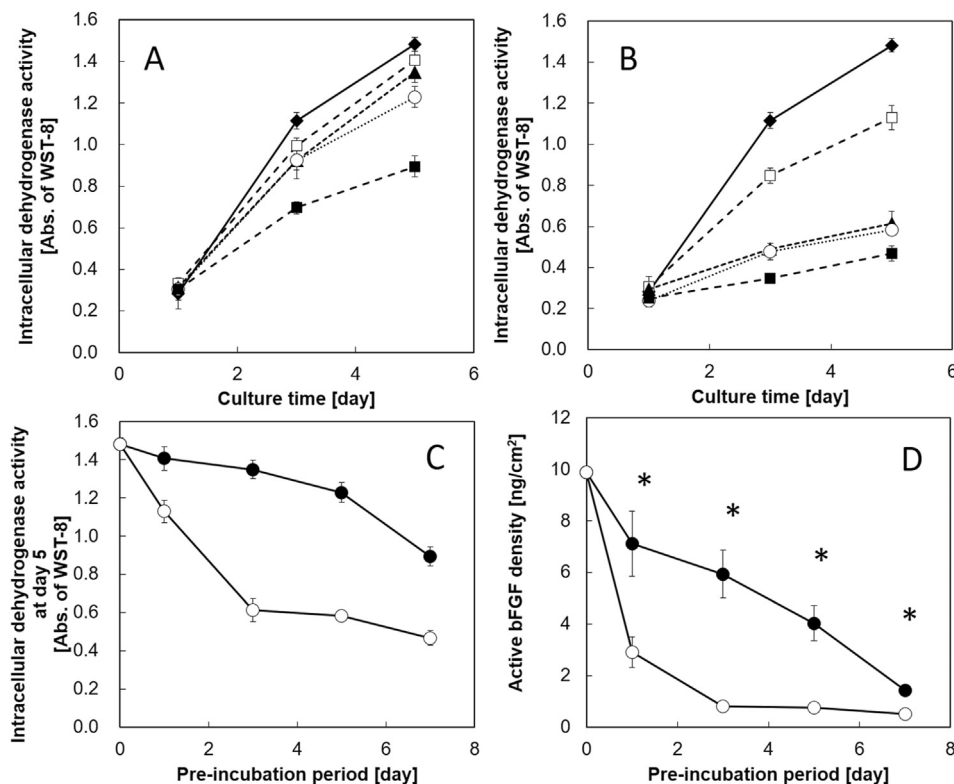


Fig. 3. Effect of bFGF pre-incubated at 37 °C on intracellular dehydrogenase activity of HUVECs and quantitative changes in biological stability of bFGF (A) Changes in intracellular dehydrogenase activity over time for immobilized bFGF pre-incubated for various periods (closed diamonds, 0 day; open squares, 1 day; closed triangles, 3 days; open circles, 5 days; closed squares, 7 days). bFGF was immobilized on hep-col films and then pre-incubated for various periods at 37 °C; n = 3. Bars indicate SD (B) Changes in intracellular dehydrogenase activity over time for bFGF solutions pre-incubated for various periods (closed diamonds, 0 day; open squares, 1 day; closed triangles, 3 days; open circles, 5 days; closed squares, 7 days). bFGF solutions were pre-incubated at 37 °C and then immobilized; n = 3. Bars represent SD (C) Relation between pre-incubation period at 37 °C and intracellular dehydrogenase activity of HUVECs after 5 days in culture (closed circles, pre-incubated immobilized bFGF; open circles, pre-incubated bFGF solution; n = 3). Bars represent SD (D) Biological stability of bFGF at 37 °C (closed circles, pre-incubated immobilized bFGF; open circles, pre-incubated bFGF solution; n = 3). Bars represent SD. *P < 0.01.

For this reason, heparin-conjugated scaffolds such as hep-col have been developed in recent years. Immobilization of bFGF on the scaffold via heparin suppresses the outflow of bFGF from the culture substrate or from the delivery site and thereby maintains a high local concentration of bFGF. Although many studies focus on the sustained release of bFGF molecules, few consider the stability of bFGF at physiological temperature.

In this study, we focused on the biological stability of bFGF immobilized via heparin under physiological conditions. When assessing the stability of bFGF, researchers usually use pre-incubate scaffolds for cell culture and divide the results of viability or the growth activity with the results of conditions without pre-incubation, whereby they calculate the rate of deactivated bFGF [7]. However, this is not a suitable method for evaluation of stability unless the amount of bFGF is directly proportional to the biological activity. In addition, the quantitation with ELISA detects both active and inactive bFGF and thus only partially reflects the biological stability of bFGF.

Previously, our group devised a calibration curve showing the relationship between the intracellular dehydrogenase activity of HUVECs after 5 days of culture and the quantity of immobilized VEGF on the hep-col films as shown in Fig. 2 B of the present paper. We succeeded to quantitatively evaluate the deactivation of immobilized VEGF and VEGF solution after pre-incubation at physiological temperature for various periods [17]. Similarly, we constructed the calibration curve showing the relationship between the intracellular dehydrogenase activity of HUVECs and the immobilized bFGF amount and thereby evaluated quantitatively the stability of bFGF immobilized via heparin.

bFGF is well-known as a heparin-binding growth factor and has a high binding affinity with heparin [18]. Complexation of bFGF with heparin through multipoint binding stabilizes the conformation in the bFGF molecule and improves resistance to thermal denaturation and enzymatic degradation, and suppresses inactivation by acidic pH [13,19–21]. In addition, a previous study has investigated the denaturation profile of bFGF with temperature using a heparin-immobilized column [22]. The study shows that as the conformational unfolding degree of bFGF molecules increases, the binding affinity between bFGF and the heparin-immobilized column decreases.

Therefore, it is considered that bFGF in the solution are denatured with the prolongation of pre-incubation period and became difficult to bind to the hep-col film. Whereas, fresh bFGF before pre-incubation are immobilized on the hep-col film at a high efficiency, and then are protected from thermal denaturation during pre-incubation by binding to heparin. Thereby, the higher density of bioactive bFGF on the hep-col film is maintained at the start of culture.

At present, it is arguable which of released bFGF and immobilized bFGF stimulated cell proliferation. Generally, the release rate of growth factors from the scaffold increases as the difference of growth factor density between the scaffold and the medium increases. Therefore, the release rate of the immobilized bFGF into the medium might increase with the increase in the immobilized bFGF density on the hep-col film. However, considering that heparin and bFGF hardly dissociate and bFGF with heparin in a culture medium stimulates cell proliferation [23], it may be that immobilized bFGF mainly promoted cell proliferation. In other word, the cell

proliferation of HUVEC shown in Fig. 3 is affected by the bioactive bFGF immobilized on the hep-col film after pre-incubation.

Therefore, it is speculated that the immobilization of bFGF to the hep-col film during pre-incubation stabilized bFGF and maintained its biological activity for about twice as long as that of the bFGF solution (Fig. 3 D).

This result shows that the immobilization of bFGF on the hep-col scaffold maintains a high local density of bFGF and improves its biological stability in culture. It also implies that the bFGF usage can be reduced in culture when a hep-col scaffold-immobilized bFGF is used. A previous study reported that bFGF with heparin promotes the growth of hESCs and maintains the undifferentiated state of hiPSCs compared with the heparin-free condition [5,24]. Therefore, if stem cells were cultured on the hep-col scaffold, bFGF in the culture medium would spontaneously immobilize and stabilize on the scaffold, thereby efficiently maintaining the stem cells and providing a stable cost-effective supply of these cells.

On the other hand, if hep-col scaffolds were implanted into a tissue, they would enable immobilization and localization of bFGF in vivo. In the in vivo environment, electrostatic interactions are generally inhibited by the high salt content, but since the binding between heparin and bFGF is relatively strong, the immobilization can be maintained [25]. Because heparin-mediated immobilization of bFGF also improves its stability, the benefits of using bFGF can be obtained more efficiently than with other immobilization methods based on electrostatic affinity.

Generally, the efficiency of adsorption of molecules in solution onto the substrate decreases as the molecular concentration increases. However, the results in Fig. 1 showed that the immobilizing efficiency of growth factors (i.e., bFGF, VEGF, and HGF) to hep-col film remained high even when using a growth factor solution having a concentration several times higher than that in the culture medium. Depending on the molecular weight of heparin, previous studies have reported that single heparin molecule can bind to multiple bFGF molecules in solution [21,26]. On the hep-col film (heparin density: about 20 $\mu\text{g}/\text{cm}^2$), at least 5000 times as many heparin molecules as the added growth factor is abundantly present. In addition, the dissociation constants of bFGF, VEGF, and HGF with heparin are 23, 12, and 165 nM, respectively, and the binding affinity is very high [18].

Therefore, hep-col scaffold is expected to show high immobilizing efficiency even when co-immobilizing multiple types of growth factors and thereby realize a synergistic effect on promoting tissue regeneration. For instance, the combination of HGF and epidermal growth factor, a representative heparin-binding growth factor, promotes the spheroid formation of hepatocytes and improves the expression and maintenance of protein production [27]. Thus, it is probable that hep-col scaffolds co-immobilized with different growth factors can upregulate the functions of implanted cells.

One study succeeded in immobilizing growth factors to biomaterials with a chemical crosslinker and an enzyme [28]. The method described in this study used covalent conjugation to establish firm bonds between growth factors and the scaffold, thereby inhibiting their initial burst from the scaffold and ensuring their sustained release, followed by scaffold biodegradation. This method applies to various growth factors since it uses a nonselective crosslinking reaction. However, immobilized growth factors may lose their bioactivity after the crosslinking reaction due to the denaturation of their bioactive sites. Other studies succeeded in spontaneously binding modified growth factors to the scaffold [29,30]. Although these modified growth factors can avoid denaturation during immobilization and are useful for various applications, their development is time-consuming and expensive.

As mentioned above, hep-col can spontaneously immobilize multiple types of growth factors (e.g., bFGF, VEGF, and HGF) due to high affinity. This implies that hep-col protects growth factors from inactivation during the immobilizing reaction and thus can save time and reduce the costs to develop modified spontaneously immobilized growth factors. Besides, hep-col is composed only of clinically used materials, suggesting its relatively high safety for in vivo applications. Based on these properties, the implanted hep-col can localize and concentrate the administered and endogenous growth factors to promote tissue regeneration at the lesion site.

Growth factors are produced in vivo during the process of tissue regeneration. For example, after partial hepatectomy, the concentrations of HGF and VEGF rise temporarily in the blood of rats [31,32]. Since hep-col can also immobilize these growth factors as described above, the following regenerative mechanism can be expected. After the angiogenesis initiation by the hep-col-immobilized bFGF, the endogenously secreted growth factors from the newly formed blood vessels are captured onto the scaffold, and from here, they promote tissue regeneration. Thus, hep-col scaffold can effectively use the native growth factors to lead tissue regeneration and reduce the costs of regenerative therapy.

In summary, hep-col is a potent scaffold material with the ability to localize, stabilize, co-immobilize, and continuously immobilize growth factors. Its advantageous effects are versatile and will thus allow further development in different fields such as regenerative medicine and biology by promoting cell functions and the stable supply of stem cells through growth factor immobilization.

5. Conclusion

We showed that heparin-conjugated collagen film immobilized bFGF, VEGF, and HGF at a high efficiency. In addition, we quantitatively evaluated the biological activity of the immobilized bFGF and the bFGF solution after pre-incubation at 37 °C with the calibration curve showing the relationship between intracellular dehydrogenase activity of HUVECs after 5 days in culture and density of the immobilized bFGF. The results showed that heparin-mediated immobilization improved the biological stability of bFGF. Therefore, heparin-conjugated collagen not only localizes different growth factors but also stabilizes bFGF at physiological temperature. Taken together, heparin-conjugated collagen is a promising potent scaffold material that can be used for further development of various applications in regenerative medicine.

Conflicts of interest

None.

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References

- [1] Onuma Y, Higuchi K, Aiki Y, Shu Y, Asada M, Asashima M, et al. A stable chimeric fibroblast growth factor (FGF) can successfully replace basic FGF in human pluripotent stem cell culture. *PLoS One* 2015;10(4):e0118931.
- [2] Horiguchi I, Urabe Y, Kimura K, Sakai Y. Effects of glucose, lactate and basic FGF as limiting factors on the expansion of human induced pluripotent stem cells. *J Biosci Bioeng* 2018;125(1):111–5.
- [3] Chen G, Gulbranson DR, Hou Z, Bolin JM, Ruotti V, Probasco MD, et al. Chemically defined conditions for human iPSC derivation and culture. *Nat Methods* 2011;8(5):424–9.

- [4] Ruel M, Laham RJ, Parker JA, Post MJ, Ware JA, Simons M, et al. Long-term effects of surgical angiogenic therapy with fibroblast growth factor 2 protein. *J Thorac Cardiovasc Surg* 2002;124(1):28–34.
- [5] Furue MK, Na J, Jackson P J, Okamoto T, Jones M, Baker D, et al. Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium. *Proc Natl Acad Sci USA* 2008;105(36):13409–14.
- [6] Chen G, Gulbranson DR, Yu P, Hou Z, Thomson JA. Thermal stability of fibroblast growth factor protein is a determinant factor in regulating self-renewal, differentiation, and reprogramming in human pluripotent stem cells. *Stem Cell* 2012;30(4):623–30.
- [7] Wu JM, Xu YY, Li ZH, Yuan XY, Wang PF, Zhang XZ, et al. Heparin-functionalized collagen matrices with controlled release of basic fibroblast growth factor. *J Mater Sci Mater Med* 2011;22(1):107–14.
- [8] Pike DB, Cai S, Pomraning KR, Firpo MA, Fisher RJ, Shu XZ, et al. Heparin-regulated release of growth factors in vitro and angiogenic response in vivo to implanted hyaluronan hydrogels containing VEGF and bFGF. *Biomaterials* 2006;27(30):5242–51.
- [9] Lee J, Yoo JJ, Atala A, Lee SJ. Controlled heparin conjugation on electrospun poly(ϵ -caprolactone)/gelatin fibers for morphology-dependent protein delivery and enhanced cellular affinity. *Acta Biomater* 2012;8(7):2549–58.
- [10] Ikegami Y, Ijima H. Development of heparin-conjugated nanofibers and a novel biological signal by immobilized growth factors for peripheral nerve regeneration. *J Biosci Bioeng* 2020;129(3):354–62.
- [11] Kanato Y, Ono S, Kitajima K, Sato C. Complex formation of a brain-derived neurotrophic factor and glycosaminoglycans. *Biosci Biotechnol Biochem* 2009;73(12):2735–41.
- [12] Mammadov R, Mammadov B, Guler MO, Tekinay AB. Growth factor binding on heparin mimetic peptide nanofibers. *Biomacromolecules* 2012;13(10):3311–9.
- [13] Gospodarowicz D, Cheng J. Heparin protects basic and acidic FGF from inactivation. *J Cell Physiol* 1986;128(3):475–84.
- [14] Vemuri S, Beylin I, Sluzky V, Stratton P, Eberlein G, Wang YJ. The stability of bFGF against thermal denaturation. *J Pharm Pharmacol* 1994;46(6):481–6.
- [15] Hou YT, Ijima H, Takei T, Kawakami K. Growth factor/heparin-immobilized collagen gel system enhances viability of transplanted hepatocytes and induces angiogenesis. *J Biosci Bioeng* 2011;112(3):265–72.
- [16] Nagai T, Ikegami Y, Mizumachi H, Shirakigawa N, Ijima H. Development of an in situ evaluation system for neural cells using extracellular matrix-modeled gel culture. *J Biosci Bioeng* 2017;124(4):430–8.
- [17] Mizumachi H, Ijima H. Measuring stability of vascular endothelial growth factor using an immobilization technique. *Adv Biomed Eng* 2013;2:130–6.
- [18] Ashikari-Hada S, Habuchi H, Kariya Y, Itoh N, Reddi HA, Kimata K. Characterization of growth factor-binding structures in heparin/heparan sulfate using an octasaccharide library. *J Biol Chem* 2004;279(13):12346–54.
- [19] Westall FC, Rubin R, Gospodarowicz D. Brain-derived fibroblast growth factor: a study of its inactivation. *Life Sci* 1983;33(24):2425–9.
- [20] Prestrelski SJ, Fox GM, Arakawa T. Binding of heparin to basic fibroblast growth factor induces a conformational change. *Arch Biochem Biophys* 1992;293(2):314–9.
- [21] Sommer A, Rifkin BD. Interaction of heparin with human basic fibroblast growth factor: protection of the angiogenic protein from proteolytic degradation by a glycosaminoglycan. *J Cell Physiol* 1989;138(1):215–20.
- [22] Shah D, Johnston TP, Mitra AK. Thermodynamic parameters associated with guanidine HCl- and temperature-induced unfolding of bFGF. *Int J Pharm* 1998;169:1–14.
- [23] Caldwell MA, Svendsen CN. Heparin, but not other proteoglycans potentiates the mitogenic effects of FGF-2 on mesencephalic precursor cells. *Exp Neurol* 1998;152(1):1–10.
- [24] Choi M, Park HH, Choi D, Han U, Park TH, Lee H, et al. Multilayer nanofilms via inkjet printing for stabilizing growth factor and designing desired cell developments. *Adv Healthc Mater* 2017;6(14):1700216.
- [25] Wissink MJ, Beermink R, Pieper JS, Poot AA, Engbers GH, Beugeling T, et al. Binding and release of basic fibroblast growth factor from heparinized collagen matrices. *Biomaterials* 2001;22(16):2291–9.
- [26] Arakawa T, Wen J, Philo JS. Stoichiometry of heparin binding to basic fibroblast growth factor. *Arch Biochem Biophys* 1994;308(1):267–73.
- [27] Ijima H, Mizumoto H, Nakazawa K, Kajiwara T, Matsushita T, Funatsu K. Hepatocyte growth factor and epidermal growth factor promote spheroid formation in polyurethane foam/hepatocyte culture and improve expression and maintenance of albumin production. *Biochem Eng J* 2009;47:19–26.
- [28] Chiu LL, Weisel RD, Li RK, Radisic M. Defining conditions for covalent immobilization of angiogenic growth factors onto scaffolds for tissue engineering. *J Tissue Eng Regen Med* 2011;5(1):69–84.
- [29] Lee H, Lim S, Birajdar MS, Lee SH, Park H. Fabrication of FGF-2 immobilized electrospun gelatin nanofibers for tissue engineering. *Int J Biol Macromol* 2016;93:1559–66.
- [30] Nishi N, Matsushita O, Yuube K, Miyakawa H, Okabe A, Wada F. Collagen-binding growth factors: production and characterization of functional fusion proteins having a collagen-binding domain. *Proc Natl Acad Sci USA* 1998;95(12):7018–23.
- [31] Lindroos PM, Zarnegar R, Michalopoulos GK. Hepatocyte growth factor (hepatopoietin A) rapidly increases in plasma before DNA synthesis and liver regeneration stimulated by partial hepatectomy and carbon tetrachloride administration. *Hepatology* 1991;13(4):743–50.
- [32] Taniguchi E, Sakisaka S, Matsuo K, Tanikawa K, Sata M. Expression and role of vascular endothelial growth factor in liver regeneration after partial hepatectomy in rats. *J Histochem Cytochem* 2001;49(1):121–30.