Action of microparticles of heparin and alginate crosslinked gel when used as injectable artificial matrices to stabilize basic fibroblast growth factor and induce angiogenesis by controlling its release

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Abstract: Alginate is an acidic polysaccharide like the glycosaminoglycans and is a candidate for use as an artificial matrix. We developed a novel alginate gel sheet that is crosslinked with heparin (H/A gel sheet) and discovered its properties of releasing biologically active basic fibroblast growth factor (bFGF), a representative member of the heparin-binding growth factors (HBGFs), for about 1 month *in vitro* and of inducing angiogenesis *in vivo*. In the present study, the H/A gel sheet was mechanically broken up to produce easily injectable 50- to 200-μm microparticles of the gel (H/A gel particles), the properties of which were analyzed. The H/A gel particles cumulatively released 2.8 times as much bFGF as the H/A gel sheet, despite both having the same amount of bFGF adsorbed onto their gels. In addition, the bFGF-adsorbed H/A gel particles released a significant amount of bFGF, which stimulated cellular growth in a culture of human umbilical venous endothelial cells for up to 5 weeks. The subcutaneous injection of the bFGF-adsorbed H/A gel particles induced the formation of numerous microvessels in the tissue surrounding the gel. These results indicate that the H/A gel particles not only stabilize bFGF by preventing the occurrence of proteolysis or denaturation but also modulate its release from the gel. Because the H/A gel particles can be easily injected into the target tissues, this artificial matrix may be useful for the local delivery of HBGFs in the treatment of ischemic arterial diseases, as well as for regenerating or constructing tissues. J Biomed Mater Res 67A: 61–68, 2003

Key words: bFGF; controlled release; injectable alginate-gel; heparin; angiogenesis

INTRODUCTION

Artificial organs or tissues are currently being made by using tissue engineering technology, which aims to regenerate damaged tissues. In the construction of tissue, artificial matrices play an important role as a cellular "scaffold" and stabilizer for the mitogenic molecules, such as growth factors.[1] For instance, collagen, polyglycolic acid, and polylactic acid are used as matrices for the construction of skin,[2] cartilage,[3] and meniscus.[4]

Heparin-binding growth factors (HBGFs), such as basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF), have powerful mitogenic effects on certain types of cells, while modulating cell proliferation and differentiation. These factors also promote wound repair and angiogenesis and could be an important tool in tissue engineering. Major obstacles to their clinical use are their short *in vivo* half-lives, instability, and unexpected adverse reaction to systemic administration.[5,6] Physiologically, HBGFs bind to the heparan sulfate glycosaminoglycans of a cell surface or extracellular matrices. The binding of HBGFs to

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glycosaminoglycans not only stabilizes the molecule against denaturation or proteolysis but also enhances their binding with cellular receptors.[7–9] Thus, heparan sulfate proteoglycans function as a stabilizer or HBGF reservoir and play an important role in regulating HBGF bioactivity in the local environment. Therefore, an artificial matrix that has similar functions to heparan sulfate proteoglycans could be very useful as a cellular scaffold and local HBGF delivery system.

Like glycosaminoglycans, alginate is a seaweed-derived acidic polysaccharide composed of β-D-mannuronic acid and α-L-guluronic acid. Because alginate is sufficiently biocompatible, it could be a candidate for an artificial matrix. We previously produced a new artificial matrix made of alginate, in the form of a transparent gel sheet that is crosslinked with covalent bonds. The alginate gel sheet is useful as a scaffold for regenerating dermis[10] nerve,[11–13] and bone.[14] To add further functions to stabilize HBGFs and to control their release from the gel, we devised a novel alginate gel sheet that is covalently crosslinked between the heparin and alginate polysaccharides with ethylenediamine (H/A gel sheet) and confirmed the sustained release of bFGF and the induction of angiogenesis.[15] Although this sheet or spongelike H/A gel is suitable for covering defects in tissue, its use is limited to relatively small areas and would likely have to be combined with some surgical procedures to be applied clinically.

In the present study, the H/A gel sheet was mechanically broken up into particles to enable its easy handling in clinical applications. The resulting gel particles can be transferred to a syringe, mixed with a solution containing HBGFs, and then injected into an objective tissue. This new type of H/A gel could release biologically active bFGF in a culture of human umbilical venous endothelial cells (HUVEC) for up to 5 weeks, whereas the injection of gel particles with bFGF induced angiogenesis *in vivo*.

MATERIALS AND METHODS

Material

Human recombinant bFGF was provided by Kaken Pharmaceutical Co. Ltd. (Tokyo). Heparin (sodium salt, grade I-A) was purchased from Sigma-Aldrich Japan (Tokyo); sodium alginate was a gift from Kimitsu Chemical Industries (Tokyo); other reagents were purchased from Wako Pure Chemicals (Osaka, Japan). HUVEC were obtained from Sanko Junyaku Co Ltd. (Tokyo). The antibody acting against tissue transglutaminase was purchased from DAKO Co. (Carpinteria, CA).

Preparation of heparin-crosslinked alginate (H/A) gel particles

H/A gel particles were obtained by mechanically breaking up H/A gel sheet. This sheet was prepared according to the method described previously.[15] Briefly, 1% wt heparin and 1% wt sodium alginate were dissolved in water and then subjected to dehydration-condensation with ethylenediamine and 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride. The resulting gel was washed with a solution containing calcium chloride and sodium chloride, washed with water, and finally freeze-dried. The lyophilized sponge-form H/A gel was broken up with a Waring blender (Waring Products, New Hartford, CT) in anhydrous ethanol for 1 min. The resulting gel particles were collected by centrifuging, and were then vacuum-dried.

Structural observations and release of heparin

H/A gel particles were observed with use of an optical microscope under both dry (in ethanol) and wet conditions [in phosphate-buffered saline (PBS)].

The release of heparin from the H/A gel particles was analyzed by assessing its anticoagulant activity. Two milligrams of H/A gel particles were placed in a cell culture insert (8-μm pore size, Nippon Bekton Dickinson Ltd Co., Tokyo) and then incubated in 0.05*M* Tris-buffered saline (TBS, pH 7.4) at 37°C. The supernatant samples were collected every 24 h for 14 days. The anticoagulant activity of the supernatant was assessed by measuring the activated partial thromboplastin time (APTT). The supernatant and normal human pooled plasma were mixed at a ratio of 1:1, and the APTT was measured by using a CA-5000 coagulation analyzer (Sysmex Co., Kobe, Japan).

Basic FGF release profile

One hundred nanograms of bFGF were dissolved in PBS containing 0.1% bovine serum albumin (BSA) and were then added to 2.5 mg of H/A gel particles or gel sheet. After each of the gel samples was held at 4°C for 12 h, it was incubated in 5 mL of PBS (10 m*M* phosphate, 0.15*M* NaCl, pH7.4) containing 0.1% BSA at 37°C under air containing 5% CO_2. All of the supernatant was collected every 24 h and stored at −80°C for the immunoassay of bFGF. Then the gels were resuspended in 5 mL of fresh buffer. The basic FGF was determined by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc., Minneapolis, MN).

Effect on the proliferation of HUVEC

HUVEC was cultured in an EBM-2 medium supplemented with 2% (v/v) fetal bovine serum, 0.04% (v/v) hydrocortisone, 0.1% (v/v) ascorbic acid, 0.1% (v/v) gentami-

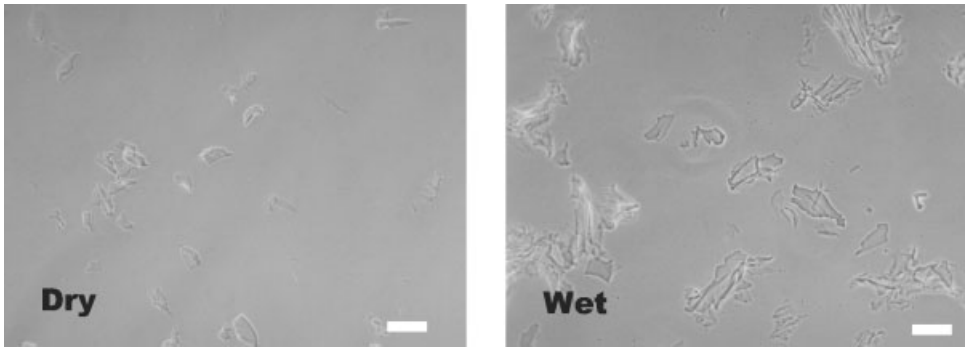


Figure 1. Microscopic appearance of H/A gel particles in ethanol (dry) or in PBS (wet). Mechanical breakdown of H/A gel sheet resulted in 50- to 200-μm particles, which expanded slightly when wet. Each gradation of the scale corresponds to 100 μm.

cin sulfate, and amphotericin-B according to the manufacture's protocol at 37°C under air containing 5% CO_2. Cells were seeded at 7500 cells/well in a 24-well flat bottom plate (Nippon Bekton Dickinson Ltd. Co., Tokyo) and were then allowed to adhere for 1 h.

Basic FGF in PBS containing 0.1% BSA (100 ng/50 μL) was adsorbed onto 2 mg of H/A and alginate gel particles. The gels were held at 4°C for 1 h and then transferred into culture inserts (8-μm pore size).

HUVEC was cultured in the presence of bFGF-adsorbed H/A or alginate gel particles for 7 days without any medium changes. After this, the bFGF-adsorbed gel particles in the culture inserts were removed and then transferred to a culture of freshly seeded HUVEC. The cellular proliferation of the resultant cells and released bFGF in the culture supernatants were assessed and compared with the negative control (culture without growth factors) and positive control [culture with the growth factor cocktail: 0.4% (v/v) bFGF, 0.1% (v/v) vascular endothelial growth factor (VEGF), 0.1% (v/v) epidermal growth factor (EGF), 0.1% (v/v) insulin-like growth factor-1 (IGF-1), and 0.1% (v/v) heparin according to the manufacturer's protocol]. The cellular proliferation was assayed by 3-(4,5-dimethylthaizol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and the bFGF was measured by using a commercial ELISA kit. This procedure was repeated for five cycles until no further cellular growth of the HUVEC was observed.

In vivo vascularization

Five milligrams of H/A-particles were transferred to a syringe under sterile conditions, and then 0.5 mL of PBS containing 0.1% BSA with 100 ng of bFGF was aspirated into the syringe and held at 4°C for 1 h. Eight-week-old male Wistar rats (Charles River Japan, Yokohama, Japan) were anesthetized with ether, and the bFGF-adsorbed gel particles were subcutaneously injected into the dorsal area. The H/A gel without bFGF and the bFGF-adsorbed alginate gel were also injected as a control. Two weeks later, the rats were euthanized by an overdose of ether. The injected gel particles and surrounding tissues were removed together, fixed in 10% neutral formalin for 1 week, sectioned at 5 μm, and then stained with hematoxylin and eosin (HE) or transglutaminase (TGase). The tissue TGase was stained by using the method described by Haroon et al.[16]

All the animal procedures were approved by the St. Marianna University School of Medicine Animal Experiment Committee.

Statistical analysis

All data are reported as means ± standard deviations (SDs). Values were evaluated by Student's t tests or the one-way analysis of variance and the subsequent Fisher's protected least-significant difference multiple-comparison test. The probability of $p < 0.05$ was taken to indicate the statistical significance.

RESULTS

Structural observation and release of heparin

The microscopic observation of H/A gel particles under both dry (in ethanol) and wet conditions (in PBS) is shown in Figure 1. The size of the gel particles varied from 50 to 200 μm under both the dry and wet conditions. However, the gel particles appear slightly expanded in PBS.

The release of heparin from the H/A gel particles into the TBS solution was assessed by measuring APTT for up to 2 weeks and was semiquantified by using standards containing different concentrations of heparin (0.01–0.5 U/mL). There was no significant release of heparin, and the daily release was estimated to be less than 0.1 U (Fig. 2).

bFGF release profile from the H/A gel particles

The *in vitro* release profile of bFGF from the H/A gel particles is shown in Figure 3. The initial burst of bFGF

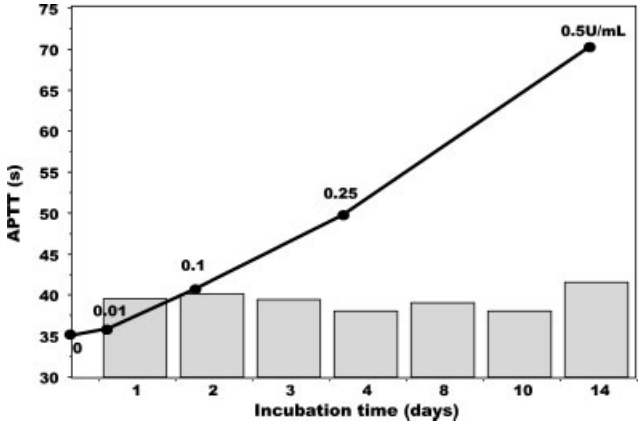


Figure 2. Five milligrams of H/A gel particles were incubated in TBS at 37°C, and the daily release of heparin was assessed by measuring the APTT of the incubation solutions (columns) and semiquantified against standards consisting of TBS solutions containing various concentrations of heparin (line). The daily release of heparin from the H/A gel particles was estimated to be <0.1 U

release from both the gel particles and gel sheet was small, after which the release was sustained for up to 28 days. The daily release of bFGF from the gel particles, however, was approximately 2–4 times greater than that from the gel sheet [Fig. 3(A)]. As shown in Figure 3(B), the gel particles cumulatively released 2.8 times as much bFGF over 7 days, compared to the gel sheet. By the 7th day of the culture, about 8% of the initially applied bFGF was released [Fig. 3(B)].

Cellular proliferation of HUVEC

To assess the biological activity of the bFGF that is released from the gel particles, we investigated the effect of the bFGF-adsorbed H/A gel particles on the proliferation of HUVEC. Although the HUVEC did not grow at all in the absence of the growth factors [Fig. 4(A)], the cells grew rapidly and reached the confluent state on the 7th day after seeding in the presence of a growth factor cocktail containing bFGF, VEGF, EGF, IGF-1, and heparin [Fig. 4(B)]. When HUVEC was cultured with bFGF (100 ng)-adsorbed H/A gel particles, the cells grew with a shape and speed similar to those cultured with growth factor cocktail during the 1st week. By repeatedly using the same gel particles, and never adding bFGF again, the stimulating effect of the HUVEC proliferation was sustained until the 5th week [Fig. 4(C)].

The results of the MTT assay showed that the proliferative activity of bFGF-adsorbed H/A gel particles was similar to that of the growth factor cocktail up until the 3rd week and then fell to about 70%, but a significantly high proliferative activity was sustained until the 5th week [Fig. 5(A)]. In contrast, the alginate gel particles could support the cellular growth of HUVEC for no more than 2 weeks. The sustained release of bFGF from H/A gel particles into the medium was observed for 5 weeks, whereas the release profile of the alginate gel particles showed an initial burst with no significant release at the 4th week [Fig. 5(B)]. The release of bFGF

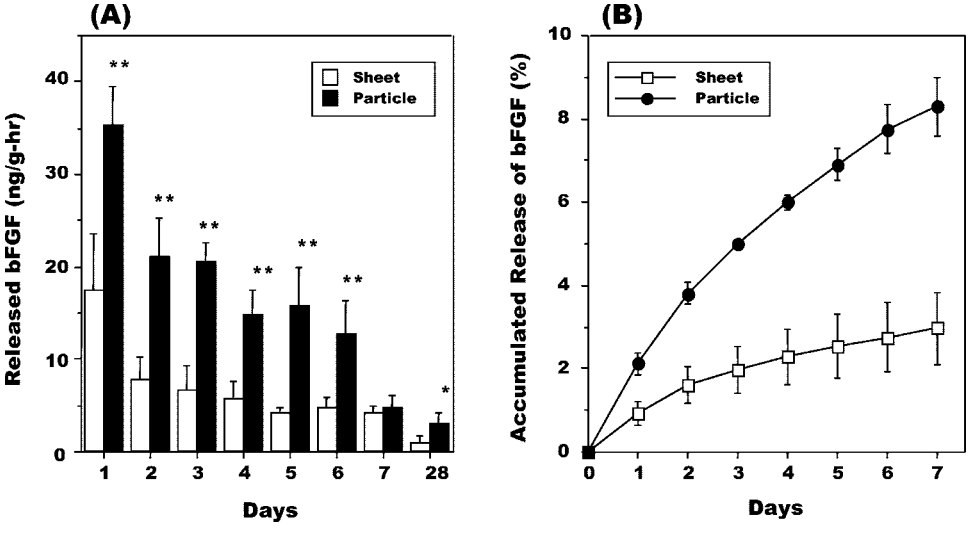


Figure 3. Comparison of bFGF release profile from H/A gel particles and gel sheet. One hundred nanograms of bFGF were adsorbed onto 2.5 mg of gel particles or gel sheet, which was then incubated in PBS containing 0.1% BSA at 37°C for up to 28 days. The concentrations of bFGF in the supernatants were measured by ELISA. Each result was from three experiments and was expressed as daily release rate (A) and accumulated release (% of the initially applied dose) (B). $*p < 0.05$, $**p < 0.01$ versus gel sheet.

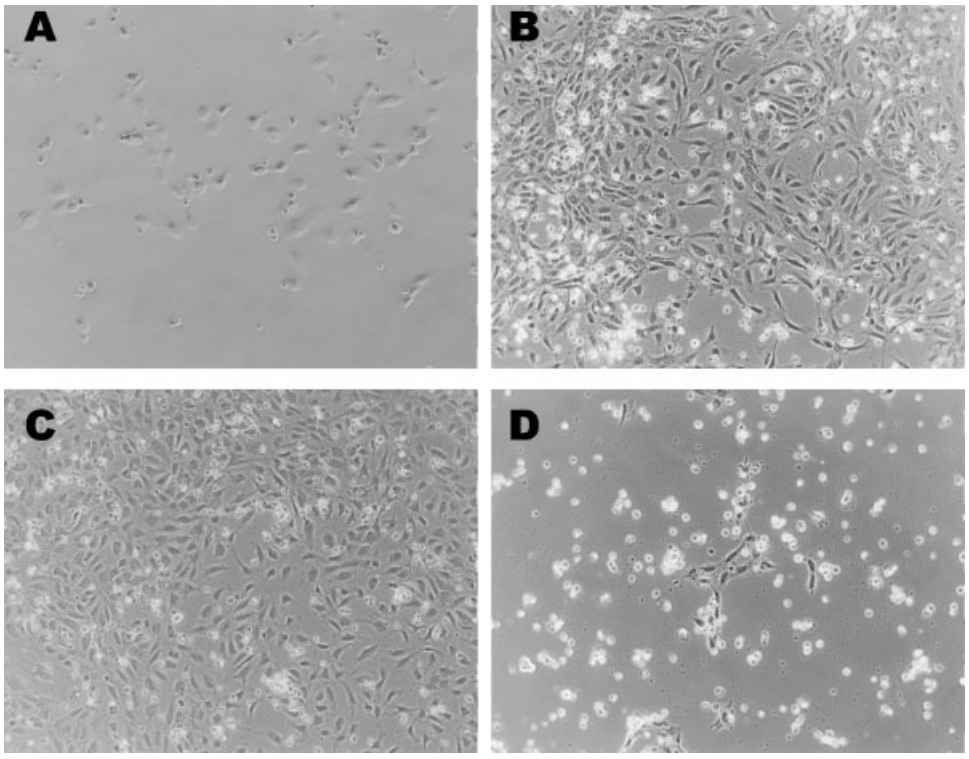


Figure 4. HUVEC was cultured for 7 days in the absence (A) or presence of the growth factor cocktail containing bFGF, VEGF, EGF, IGF, and heparin (B). HUVEC grew rapidly in the presence of the growth factor cocktail, but no cellular proliferation was obtained in the absence of growth factors. The HUVEC was also cultured with bFGF-adsorbed H/A gel particles or alginate gel particles (100 ng of bFGF/2 mg of gel particles) for 7 days and, at the end of culture, the same gel particles were used for the newly prepared HUVEC culture. This process was repeated for five cycles. The bFGF-adsorbed H/A gel particles supported the cellular growth of HUVEC until the 5th week (C), whereas no significant growth of HUVEC was observed with the bFGF-adsorbed alginate gel particles at the 5th week (D). Original magnification ×100.

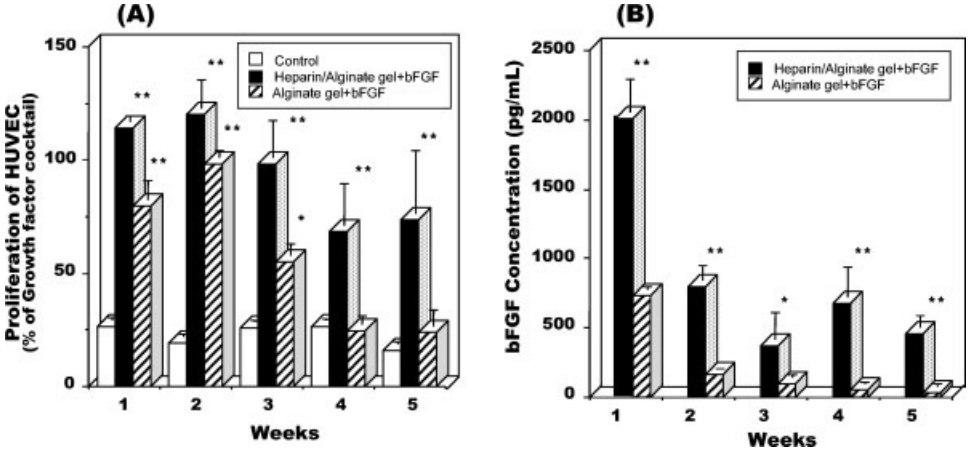
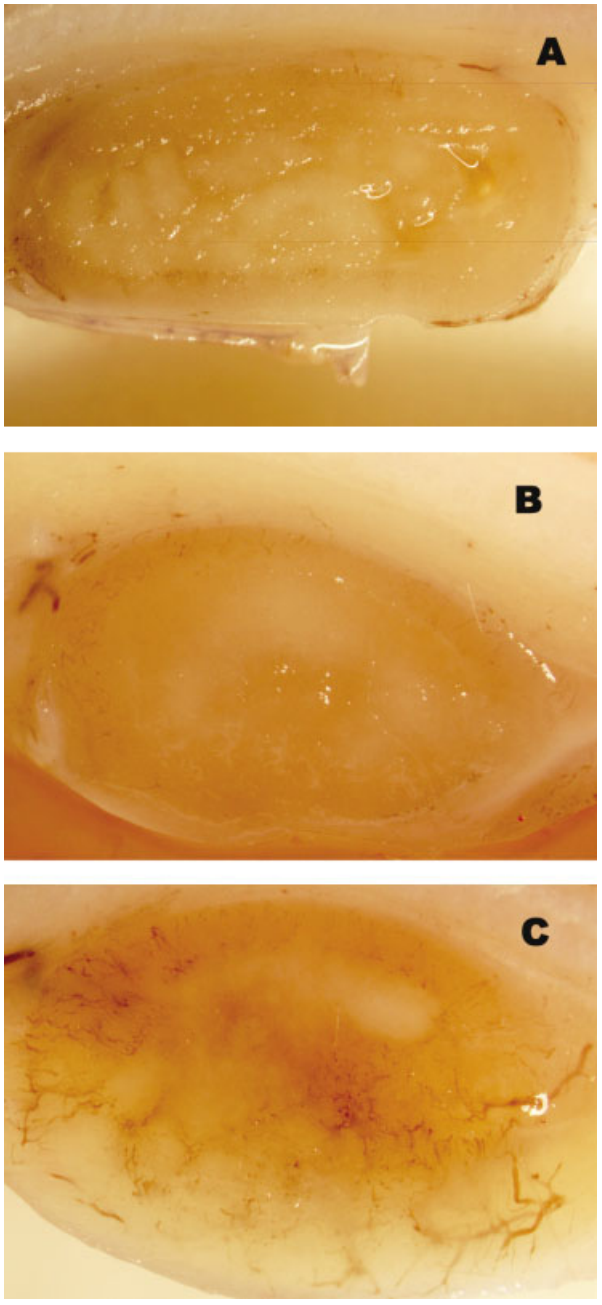


Figure 5. The proliferation of HUVEC by bFGF-adsorbed H/A gel particles was assessed by MTT assay and expressed as a percentage of the cellular growth in the presence of the growth factor cocktail (A). The stimulation of the cellular proliferation of HUVEC was observed for up to 5 weeks. $^{*}p < 0.05$, $^{**}p < 0.01$ versus control. The significant release of bFGF to stimulate HUVEC growth from the H/A gel particles was confirmed by ELISA for up to 5 weeks, whereas the release of bFGF from alginate gel particles decreased at the 4th week (B). The release of bFGF from H/A gel particles was higher than that from the alginate gel particles over 5 weeks. $^{*}p < 0.05$, $^{**}p < 0.01$ versus alginate gel particles. For each result, the mean ± standard deviation was taken from three experiments.



from the H/A gel particles was higher than that from the alginate gel particles over 5 weeks.

Induction of angiogenesis by H/A gel particles with bFGF

The injection of 5 mg of H/A gel particles containing 100 ng of bFGF into the subcutaneous space induced abundant neovascularization in the tissue surrounding the gel particles, compared with the H/A gel without bFGF or the bFGF-adsorbed alginate gel that does not contain heparin [Fig. 6(A–C)]. The histological examination of the tissue surrounding the H/A gel particles showed the formation of granulation tissue that consists of numerous microvessels confirmed by TGase staining, fibroblast-like cells, and macrophages [Fig. 7(A and B)].

DISCUSSION

We previously reported on a novel artificial matrix covalently crosslinked between alginate and heparin. This spongelike or sheet-type gel released biologically active bFGF for up to 1 month. In the present study, the H/A gel sheet was mechanically broken up into particles with a size of between 50 and 200 μm. The resultant gel particles can be transferred to a syringe, mixed with HBGF solutions to adsorb growth factors, and then injected into an objective tissue.

To clarify the effect of the mechanical breakup of the

Figure 6. Neovascularization in subcutaneous tissue by the injection of bFGF-adsorbed H/A gel particles. The cross section of the subcutaneous tissues shows the induction of angiogenesis by bFGF-adsorbed H/A gel particles in the injection site (C) compared to the control (A) or to the bFGF-adsorbed alginate gel particles (B).

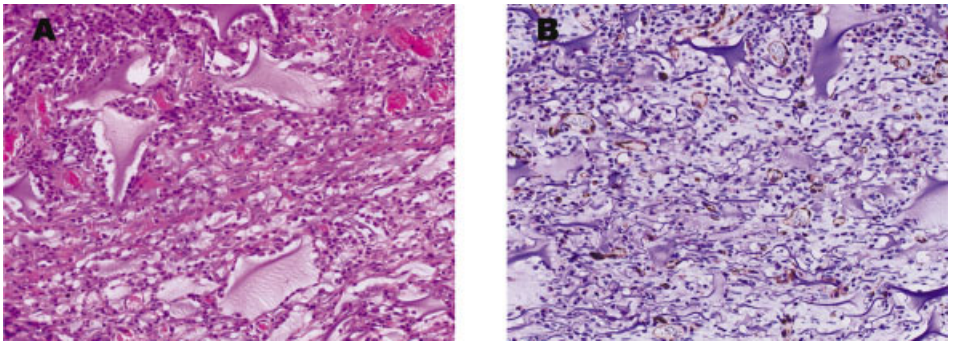


Figure 7. The histological examination of the subcutaneous tissue surrounding the H/A gel particles. The formation of numerous microvessels and cellular infiltration were observed: (A) HE staining; (B) immunological localization of TGase). Original magnification ×200.

gel on the binding characteristics of HBGF, the binding capacity and release profile of the gel particles for bFGF were investigated *in vitro*. The bFGF release profile showed that the H/A gel particles released more bFGF than the H/A gel sheet for up to 4 weeks. The higher release of bFGF from the H/A gel particles might be a result of the increased binding capacity caused by the increase in the binding surface area. Although the *in vitro* accumulated release of bFGF from the H/A gel particles by the 7th day was about 8% of the initially applied dose, after that the release was sustained for up to 4 weeks.

In addition, the bioactivity of the released bFGF was determined in the culture of HUVEC. The release of bFGF in the culture supernatant and the proliferation of HUVEC were observed for up to 5 weeks. It is of interest that the bFGF-adsorbed H/A gel particles supported the cellular growth of HUVEC for 3 weeks, in much the same way as when provided with a freshly prepared growth factor cocktail every week with 100 ng of bFGF adsorbed onto the gel particles. These findings indicate that the bFGF was bound to the heparin molecules in the H/A gel particles and was released slowly without any loss of bioactivity for about a month. The binding of the bFGF to heparin in the gel may not only prevent denaturation or proteolysis of the molecule by proteases, high temperature, or low pH[17,18]) but may also provide a controlled release system.

The stabilization and controlled release of the bFGF was confirmed *in vivo* by the induction of neovascularization in the targeted tissue. The subcutaneous injection of bFGF-adsorbed H/A gel particles induced the formation of numerous microvessels, which expressed TGase. In contrast, no siginificant neovascularization was observed by the injection of bFGF-adsorbed alginate gel particles that did not contain heparin. The previous report showed that TGase was expressed in the newly generated blood vessels and might play a role in wound repair.[16]

Recently, the effectiveness of the local administration of bFGF was reported in the treatment of ischemic arterial diseases. The local perivascular delivery of bFGF by using heparin-sepharose alginate pellets in patients undergoing coronary bypass surgery has proven useful for preventing anginal pain and reducing the ischmeic myocardial area, although this bFGF delivery system requires a large amount of bFGF (100 μg) and multiple linear incisions in the epicardial fat surrounding the target vessels.[19] The H/A gel particles can be easily handled without the need for complicated procedures because they can be injected into almost any location. With this simple administration, we can expect not only a reduced operation time but also the prevention of bacterial infections in clinical situations. Therefore, H/A gel particles could be used to deliver bFGF to an ischemic zone with the use of an easier procedure and might be more effective for such pathological conditions. In addition, the delivery of bFGF through the injection of H/A gel particles could be applied to the treatment of the peripheral ischemic ulcers or gangrene caused by atherosclerosis, Burger's disease, and diabetes mellitus.

Because bFGF has been used for the healing of wounds after the full-thickness excision of skin,[20] the *de novo* formation of adipose tissue,[21] the protection of neurons,[22,23] and the proliferation of ES cells,[24] the H/A gel particles could prove to be an important tool for tissue repair and construction by tissue engineering. Further study is necessary to clarify the usefulness of H/A gel for the delivery of other heparin-binding growth factors, such as HGF and VEGF, and for combination with cellular transplantation.

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