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# Vascularization in vivo caused by the controlled release of fibroblast growth factor-2 from an injectable chitosan/non-anticoagulant heparin hydrogel

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**Abstract**

Addition of various heparinoids to the lactose-introduced, water-soluble chitosan (CH-LA) aqueous solution produces an injectable chitosan/heparinoid hydrogel. In the present work, we examined the capability of the chitosan/non-anticoagulant heparin (periodate-oxidized ($IO_4$-) heparin) hydrogel to immobilize fibroblast growth factor (FGF)-2, as well as the controlled release of FGF-2 molecules from the hydrogel in vitro and in vivo. The hydrogel was biodegraded in about 20 days after subcutaneous injection into the back of a mouse. When the FGF-2-incorporated hydrogel was subcutaneously injected into the back of both mice and rats, a significant neovascularization and fibrous tissue formation were induced near the injected site. These results indicate that the controlled release of biologically active FGF-2 molecules is caused by biodegradation of the hydrogel, and that subsequent induction of the vascularization occurs.


*Keywords:* Chitosan; Heparinoid; Fibroblast growth factor-2; Chitosan/heparinoid hydrogel; Non-anticoagulant heparin; Vascularization

## 1. Introduction

It has been recognized that growth factors contribute to tissue regeneration at various stages of cell proliferation and differentiation [1,2]. And although many studies using growth factors have been carried out in the field of tissue regeneration, its use has not always been achieved successfully in vivo [3]. One of the reasons for this difficulty is the high diffusibility and the very short half-life time of growth factors in vivo to retain their biological activity. Thus, it is required to enhance their activities in vivo in order to apply growth factors in tissue regeneration.

Among the fibroblast growth factors, FGF-2 is well characterized [2]. It is a potent modulator of cell proliferation, motility, differentiation, and survival, as well as plays an important role in normal regeneration processes in vivo, i.e. embryonic development [4,5], angiogenesis [6], osteogenesis [7,8], chondrogenesis [9], and wound repair [10]. FGF-2 is known to be stored in various sites of the body, interacting with glycosaminoglycans such as heparin and heparan sulfate of the extracellular matrix [11,12].

FGF-2 specifically binds to heparin and heparan sulfate with a high affinity, and both its mitogenic activity and biological stability are modulated by heparin and heparan sulfate [13,14]. Heparin enhances the mitogenic activity of FGF-1 and FGF-2 [11–14]. Heparin and heparan sulfate also protect FGF-2 from inactivation by acid and heat, as well as from degradation by proteases [11,12]. Other studies have showed evidence that heparin and heparan sulfate

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serve as co-factors to promote binding of FGF-2 to high-affinity receptors, enhancing its activity [15,16]. Based on this in vivo storage mechanism, controlled release of the heparin-binding growth factors has been described from heparin-carrying polystyrene-bound collagen substrata [17], acidic gelatin hydrogels [3], alginate gels containing heparin [18], and photocrosslinkable chitosan hydrogels [19].

We have previously reported the preparation of a novel water-soluble chitosan by introducing lactose moieties [20–22]. The material is a viscous solution and is easily gelled upon mixing with a non-anticoagulant ($IO_4$-) heparin [23] solution, resulting in an injectable hydrogel. The purpose of the present study has been to evaluate the chitosan/$IO_4$-heparin hydrogel as a carrier material for controlled release of FGF-2, both in vitro and in vivo. Also, the in vivo degradability of the hydrogel, as well as the effect of FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel on vascularization and fibrous tissue formation has been examined.

## 2. Materials and methods

### 2.1. Preparation of an FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel and release of FGF-2 from the hydrogel in vitro

Water-soluble chitosan molecules (CH-LA) have been prepared as previously reported [20]. The chitosan used in this study had a molecular weight of 800–1000 kDa with a deacetylation ratio of 0.8 (Yaizu Suisankagaku Industry Co., Ltd., Shizuoka, Japan). Lactose (lactobionic acid) moieties have been introduced through the condensation reaction with amino groups of the chitosan. The introduction of lactose resulted in a water-soluble chitosan at neutral pH values. Non-anticoagulant ($IO_4$-) heparin was prepared using native heparin from porcine intestine, as has been reported previously [24].

One ml of phosphate-buffered saline (PBS) containing 50 μg/ml FGF-2 (Fiblast; Kaken Pharmaceutical Corp., Tokyo, Japan) and 1 mg/ml $IO_4$-heparin was mixed into 1 ml of 40 mg/ml CH-LA aqueous solution (final solution; 20 mg/ml CH-LA) using vortex. The obtained hydrogel (50 μl) was then spotted on the center of a well in a 24-multiwell tissue culture plate. The hydrogel was rinsed twice with medium 199 (Life Technologies Oriental, Tokyo, Japan) supplemented with 10 wt% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 μg/ml penicillin G and 100 μg/ml streptomycin) and gently shaken in 1 ml of the medium on a rotary shaker at room temperature for indicated time periods. The medium was changed every day. The concentration of FGF-2 released in the medium was determined using an ELISA assay containing heparin beads (Sigma-Aldrich Japan Corp., Tokyo, Japan) [25], and the $IO_4$-heparin concentration was determined using a dimethylmethylene blue method described by Farndale et al. [26].

### 2.2. Effect of an FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel on HUVEC growth

Human umbilical vein endothelial cells (HUVECs; Takara Biochemical Corp., Ohtsu, Japan) were cultured in medium-199 supplemented with 10 wt% heat-inactivated FBS, antibiotics, and 10 ng/ml FGF-2. The cells used in this work were all between the fourth and eighth cell cycle passage.

Fifty microliter of the CH-LA solution containing FGF-2 (1.25 μg) and $IO_4$-heparin (25 μg) was spotted into the center of a well of a 24-multiwell culture plate. The FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel was washed with 1 ml of the medium at indicated days on a rotary shaker at room temperature. The medium was changed every day. HUVECs were then plated into the well at an initial density of 15,000 cells/well in medium-199 supplemented with 10 wt% heat-inactivated FBS and antibiotics without FGF-2 and incubated for a period of 3 days. After incubation, the used medium was replaced with 100 μl of fresh medium including 10 μl of WST-1 reagent (Cell Counting Kit, Dojindo Co. Ltd., Kumamoto, Japan), and the optical density (OD) was read at 450 nm in an Immuno Mini plate reader (Nunc InterMed, Tokyo, Japan) after 1 h incubation at 37°C.

In order to examine the effect of partial degradation of the hydrogel, a 7 days washed FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel was treated with a solution containing 1 mg/ml of chitinase (chitinase-RS; Seikagaku Corp., Tokyo, Japan) and 2 mg/ml of chitosanase (chitosanase-RD; Seikagaku Corp.) for an indicated time period. HUVECs were then plated into the well, including the washed and enzyme-treated FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel. The cells were incubated in medium-199 supplemented with 10% heat-inactivated FBS and antibiotics without FGF-2, but containing chitinase (1 mg/ml) and chitosanase (2 mg/ml). These enzymes in the medium did not affect HUVEC growth, but did partially degrade the chitosan hydrogel. After 3–6 days incubation of the hydrogel in this medium, many cracks in the hydrogel, as well as small fragments of the hydrogel in the medium, were observed, suggesting partial degradation of the hydrogel.

### 2.3. Estimation of in vivo degradation of trypan blue-incorporated chitosan hydrogels

In vivo degradation of a chitosan/$IO_4$-heparin hydrogel has been examined by a decrease of trypan blue (acidic dye; Sigma-Aldrich, Japan) in the trypan blue-incorporated hydrogel. Since trypan blue was not

released from a trypan blue-incorporated chitosan/$IO_4$-heparin hydrogel in vitro (data not shown), a decrease of trypan blue in the hydrogel in vivo should be identical to the biodegradation of the hydrogel. The hydrogel (100 μl) containing 100 μg/ml of the trypan blue was injected into both the right and left sides of the back subcuits of mice, each weighing around 30 g (C57BL/6, female, 10-week-old, Crea Japan Inc., Tokyo, Japan) 2 cm apart from the tail root. The mice were sacrificed at 2, 5, 9, 14 and 21 days after the hydrogel injection and subsequently the tissue including the injected hydrogels was removed. The blood around the removed tissue was rinsed and the remaining hydrogel was treated with 0.5 ml of 100 mM $NaNO_2$ at pH 3.0 and centrifuged at 2000 rpm. The trypan blue in the supernatant was determined by measuring the $OD_{640}$.

### 2.4. Neovascularization induced by an FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel

An FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel (100 μl) was injected carefully into both the right and left sides of the back subcuits of the mice 2 cm apart from the tail root. To evaluate neovascularization in the mouse back subcuis, the mice were sacrificed at the indicated day after hydrogel injection, and subsequently the weight of tissue hemoglobin was determined as previously reported [19]. Briefly, a fixed area of the subcutaneous tissue and skin around the injected site of the hydrogel was removed and rinsed with PBS. The removed tissues were immersed in red blood cell lysing reagent (Sigma and Aldrich) and then minced using a scalpel. Hemoglobin was extracted from the minced tissue during 24 h at 4°C, and quantified using a total hemoglobin assay kit (Sigma and Aldrich).

### 2.5. Histological examination

The removed tissues including the hydrogel from rats (male Sprague Dawley rats; Crea Japan Inc., Tokyo, Japan) were fixed in a 10% formaldehyde solution, embedded in paraffin and sectioned. The sections were stained with hematoxylin-eosin (H&E) reagent. In each section, a randomized area (microscopic fields, 100 ×) showing the largest capillary density, was photographed, and the number of mature vessels containing erythrocytes per microphotograph was counted (Fig. 7). Animal experiments were carried out according to the protocol approved by the Animal Experimentation Committee of the National Defense Medical College (Saitama, Japan).

### 2.6. Statistical analysis

Statistical analysis was carried out by an unpaired Student's $t$ test using means and standard error determinations for each experimental group.

## 3. Results

### 3.1. Release of incorporated FGF-2 from chitosan/$IO_4$-heparin hydrogels and effect of FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel on HUVEC growth

Fig. 1 shows the release profiles of FGF-2 and $IO_4$-heparin from the chitosan hydrogels at room temperature in the medium. Only a minor amount of $IO_4$-heparin released from the hydrogel, and approximately 20% of FGF-2 released within the first day, followed by no further substantial release after that.

FGF-2 is well known to be a stimulating factor for endothelial cell growth. The FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel was able to stimulate HUVEC growth, but washings of the hydrogels with the medium for longer than 5 days resulted in a loss of ability to stimulate HUVEC growth (Fig. 2A). Washing of the FGF-2-incorporated hydrogels with the medium (changed once a day) for 5 days appeared to result in a loss of release of the FGF-2 into the culture medium, in agreement with the result found in Fig. 1.

In order to examine the stability of the FGF-2 molecules in the hydrogel, HUVECs were cultured with a 7 days-washed FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel in the presence of both chitinase and chitosanase. HUVECs grew normally in the medium containing the enzymes (data not shown). The enzymes did cause many cracks in the chitosan hydrogel, as well as small fragments of the hydrogel in the medium after 3 days incubation (data not shown). Although 7 day-washed FGF-2-incorporated hydrogels completely lost the ability to stimulate HUVEC growth, the stimulating activity on HUVEC growth highly recovered upon treating the FGF-2-incorporated hydrogel with the enzymes (Fig. 2B). These results indicated that (i) partial degradation of the FGF-2-incorporated hydrogels did

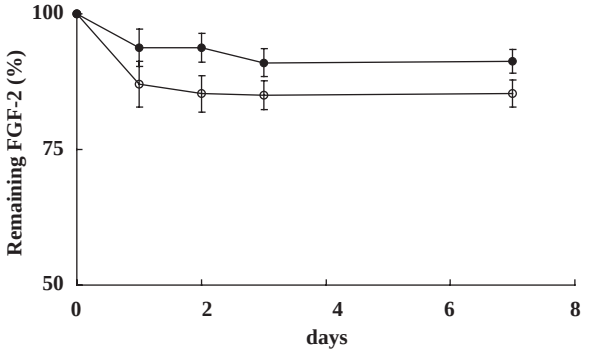


Fig. 1. Time course of remaining incorporated FGF-2 and $IO_4$-heparin in the hydrogel. The concentration of released molecules into the culture medium was quantified, as described in Materials and Methods. $IO_4$-heparin (●) and FGF-2 (○). Amounts of initially incorporated molecules in the chitosan hydrogel were defined as 100%. Each data point represents the mean ± SD of tetraplicate determinations.

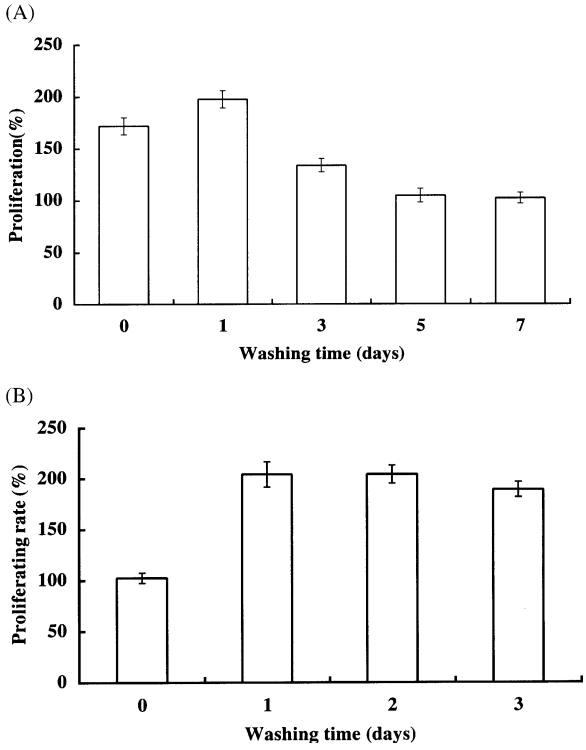


Fig. 2. Effect of FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel on the HUVEC growth in vitro. FGF-2-incorporated chitosan/$IO_4$-heparin hydrogels on the center of culture plate were washed with for indicated period of time (panel A) and HUVECs were cultured. The grown cells were measured after incubation for 3 days. In panel B, the hydrogels pre-washed for 7 days were further washed with the culture medium containing chitinase and chitosanase to partly degrade the hydrogel for the indicated periods and HUVECs were subsequently cultured for 3 days in the medium containing the chitinase and chitosanase. The cells incubated with the chitosan/$IO_4$-heparin hydrogel (without FGF-2) have been defined as 100%. The cell growth data were then calculated as a percentage, representing the mean ± SD of tetraplicate determinations.

occur in the presence of chitinase and chitosanase, and (ii) the FGF-2 within the hydrogel retained its biologically active form after a 7 days hydrogel-washing period.

### 3.2. *In vivo degradation of trypan blue-incorporated chitosan/$IO_4$-heparin hydrogels*

In vivo degradation of a chitosan/$IO_4$-heparin hydrogel (100 μl per mouse) was examined using trypan blue-incorporated chitosan/$IO_4$-heparin hydrogels. Namely, the trypan blue molecules interacting with the hydrogel would not release without degradation of the chitosan hydrogel itself [19]. The dye amount in the trypan blue-incorporated hydrogel, injected into the back of mice, decreased with implantation time (Fig. 3). About 80%, 60% and 30% of the dye molecules were retained in the implanted chitosan hydrogel on day 2, 5, and 14, respectively. However, many blue gel fragments near the implanted sites were observed on days 9, 14, and 21.

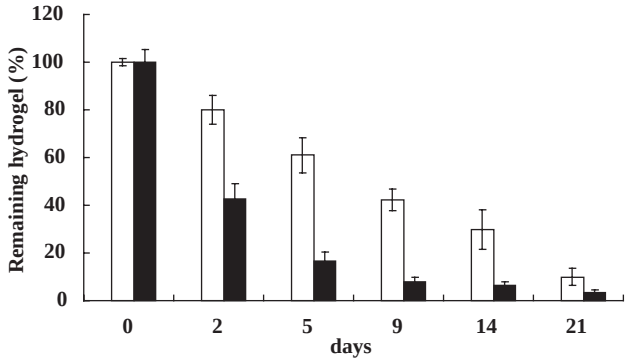


Fig. 3. Biodegradation of trypan blue-incorporated chitosan/$IO_4$-heparin hydrogels. Degradations in vivo of trypan blue-incorporated chitosan/$IO_4$-heparin hydrogels (white bars) and trypan blue-incorporated chitosan alone (black bars) in the back subcuits of mice were examined, as described in Materials and Methods. The amount of initially incorporated trypan blue is defined as 100%. The data represent the mean ± SD of six determinations (three mice for each point).

Thus, the injected hydrogel was partially biodegraded in vivo in about 21 days after implantation into the back of mice.

### 3.3. *Neovascularization induced by FGF-2-incorporated chitosan/$IO_4$-heparin hydrogels*

Tissue hemoglobin in subcutaneous tissue in mice around the injected sites of the FGF-2-incorporated chitosan/$IO_4$-heparin hydrogels was measured in order to evaluate the controlled release of FGF-2 from the hydrogels and its neovascularization. Fig. 4A shows the concentration effect of FGF-2 incorporated in the hydrogel on neovascularization. More than 1 μg of FGF-2 in the hydrogel (100 μl) has a positive effect to induce neovascularization in vivo in a concentration-dependent manner.

Fig. 4B shows the time course of neovascularization in mice induced by the FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel (200 μl). The tissue hemoglobin amount increased until 4–8 days after injection of an FGF-2-incorporated hydrogel and then slightly decreased from day 14. Since injections of an FGF-2 solution, $IO_4$-heparin solution, an FGF-2/$IO_4$-heparin solution, FGF-2/chitosan, and a chitosan/$IO_4$-heparin hydrogel did not show significant neovascularizations in mice, co-addition of FGF-2 into the chitosan/$IO_4$-heparin hydrogel resulted in a significant enhanced vascularization effect.

### 3.4. *Histological observations on the vascularization effect*

To confirm the effect of an FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel on the vascularization

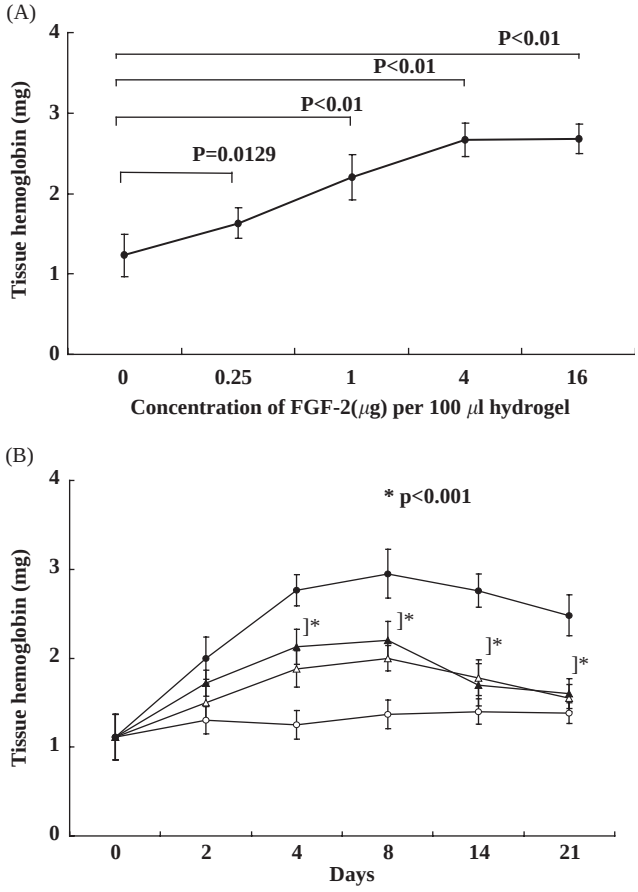


Fig. 4. Vascularization induced by FGF-2-incorporated chitosan and $IO_4$-heparin hydrogel. Effect of FGF-2 concentration incorporated in chitosan/$IO_4$-heparin hydrogels on neovascularization (A). Time course of neovascularization induced by FGF-2-incorporated chitosan/$IO_4$-heparin hydrogels (B). Neovascularization induced by an injection of FGF-2-incorporated chitosan and $IO_4$-heparin hydrogel (●), FGF-2-incorporated chitosan (▲), chitosan/$IO_4$-heparin hydrogel (△), and chitosan alone (○) for indicated days was evaluated as an amount of tissue hemoglobin. The data represent the mean ± SD of eight determinations (four mice for each point).

and fibrous tissue formation in rats, histological observations of tissue near the injected hydrogel have been carried out with H&E staining. Fig. 5 clearly shows a considerable amount of the FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel and chitosan/$IO_4$-heparin hydrogel remaining with numerous neutrophils migrated into the gel 2 weeks after injection. The FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel remaining areas in the removed tissue at days 4, 8 and 14 days after injection were covered by glossy smooth pleura. The hydrogels were also encapsulated by fibrous tissue and covered with regenerated thick pleura. These fibrous tissues were not observed after injections with chitosan alone and chitosan/$IO_4$-heparin hydrogel, although small fibrous tissues were observed around FGF-2/chitosan-injected sites.

The capillary number per microphotograph ( × 100) in tissues near the injected sites was determined in Fig. 7. Representative microphotographs of tissue around each injected site at day 8 after injection are shown in Fig. 6 and the capillary number was determined as in Fig. 7. Numerous mature vessels containing erythrocytes were observed around the injected FGF-2-incorporated chitosan/$IO_4$-heparin hydrogels already from day 4 onwards. The neovascularization was however not observed at day 2 after injection of the FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel (data not shown).

## 4. Discussion

We have reported that a photocrosslinkable chitosan hydrogel shows the ability of a controlled release of various growth factors acting as novel carriers and inducing neovascularization in vivo [19]. However, the photocrosslinkable chitosan hydrogel is not injectable due to the requirement of UV irradiation in order to obtain the crosslinked gel. In the present work, we have prepared an injectable FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel, and evaluated its effect on the vascularization and fibrous tissue formation using mice and rats. Our main conclusions from this work are (i) FGF-2 molecules incorporated into the chitosan/$IO_4$-heparin hydrogel gradually release upon biodegradation of the hydrogel and (ii) FGF-2-incorporated chitosan/$IO_4$-heparin hydrogels show a substantial effect to induce vascularization and fibrous tissue formation.

Heparin is clinically used as an antithrombotic agent, but a high dose use is limited by its strong intrinsic anticoagulant property, causing severe bleeding complications [24]. If heparin could be modified to minimize its strong anticoagulant property and also to enhance growth factor activity and stability, then such a modified heparin would be a very useful drug in wound repair and regeneration. Periodate-oxidized ($IO_4$-) heparin [27] is known for not having a specific pentasaccharide structure to interact with antithrombin III [13,14], and therefore its anticoagulant activity is much lower than native heparin [27]. Therefore, the $IO_4$-heparin, as a non-anticoagulant heparin, was used to prepare chitosan/heparinoid hydrogels in this work.

It is recognized in polymer chemistry that positively and negatively charged polymers ionically interact with each other [3,20]. Basic chitosan molecules complexed with acidic molecules ($IO_4$-heparin and other heparinoids) form a hydrogel through ionic interactions. It seems likely that polypeptides such as FGF-2, once ionically complexed with chitosan or acidic molecules, are not released from the hydrogel. Since the chitosan and $IO_4$-heparin hydrogel is biodegradable in vivo (Fig. 3), incorporating polypeptides in the hydrogel will provide an excellent controlled release system.

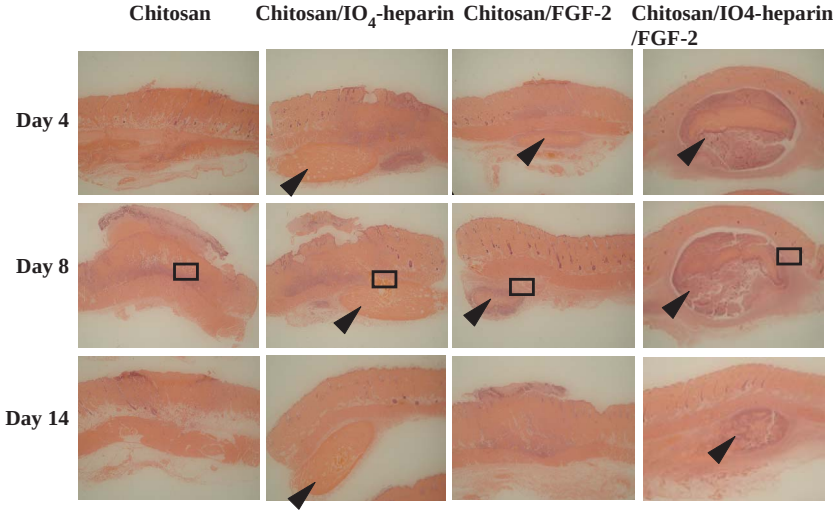


Fig. 5. Histological examination for the various chitosan hydrogel-injected sites of rats on days 4, 8, and 2 weeks after the injection. Each photograph (original magnification $\times 5$) is representative of six tissue samples stained with hematoxyline and eosin (H&E) in each group. Black arrows show the remaining hydrogel in the tissue, and white squares show the site for the microphotographs (Fig. 6).

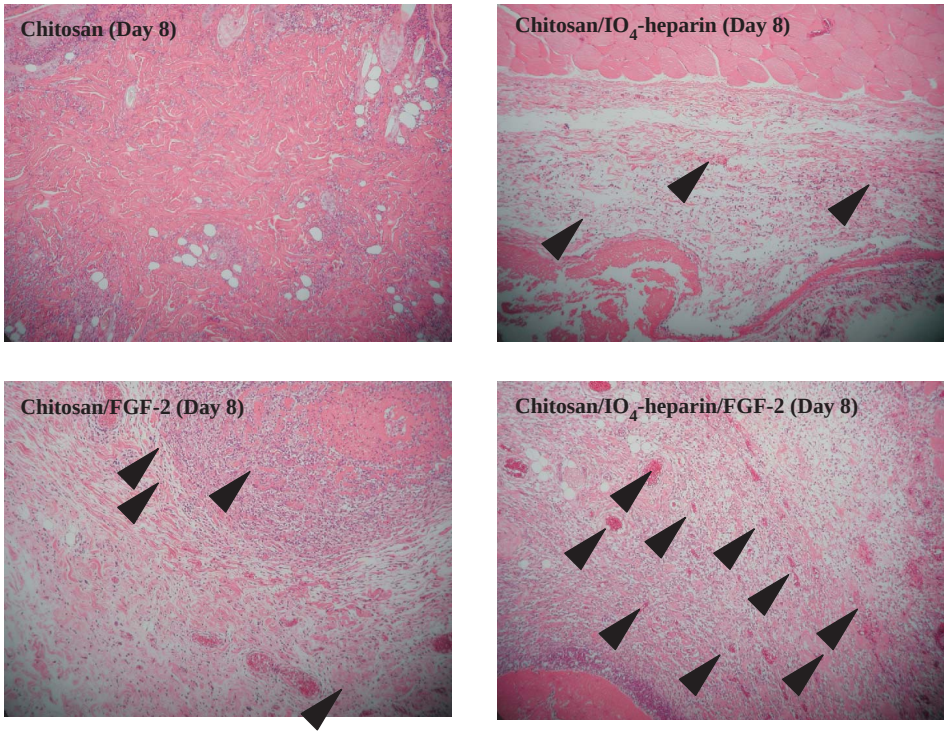


Fig. 6. Histological examination of the various chitosan hydrogel-injected sites in rats at day 8 after injection. Each microphotograph (original magnification $\times 100$) is shown as a square area in Fig. 5 and is representative of six tissue samples stained with hemotoxyline and eosin (H&E). Black arrows show mature vessels containing erythrocytes.

Although the stimulating activity of FGF-2-incorporated chitosan/$IO_4$-heparin hydrogels in HUVEC culturing was completely lost upon washing the hydrogels with culture medium for 7 consecutive days, the activity was recovered by subsequent treating of the FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel with chitinase and chitosanase during cell culturing. Thus, remaining FGF-2 molecules in the hydrogel retained their active form to accelerate cell proliferation even when electrostatically complexed with the hydrogel. Furthermore, the presence of $IO_4$-heparin in the hydrogel appeared to even enhance the activity of an FGF-2 molecule and to stabilize its structure in vitro. In fact, heparin and $IO_4$-heparin are well known to

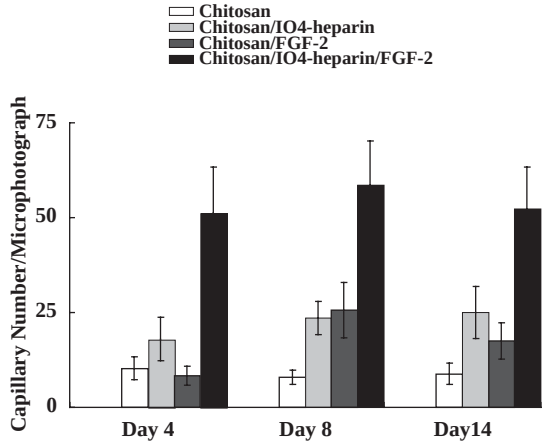


Fig. 7. Effect of FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel injection on the vascularization in vivo. The number of capillaries was counted using the microphotograph of each section ($n = 6$) considered to show the largest mature capillary density.

enhance FGF-1 and FGF-2 activities and to protect the molecules from inactivation by acids, heat, and degradation by proteases [11,12].

When FGF-2-incorporated chitosan/$IO_4$-heparin hydrogels were subcutaneously injected into the back of mice for 4 days, neovascularization was induced near the injected site of the hydrogels in a concentration-dependent manner of FGF-2 (Fig. 4). Neovascularization induced by an FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel showed a maximum from day 5 to 9, after which a slight decrease of neovascularization took place. No significant vascularization was observed by injection of FGF-2 alone, $IO_4$-heparin alone, FGF-2 plus $IO_4$-heparin, FGF-2/chitosan, and chitosan/$IO_4$-heparin hydrogel. It is suggested that injection of FGF-2 without a hydrogel carrier causes too rapid diffusion of FGF-2 molecules from the injected site to induce any vascularization effect. It is thus proposed that the FGF-2-incorporated chitosan/$IO_4$-heparin hydrogel may be a promising new biomaterial to induce vascularization and fibrous tissue formation in ischemic limbs.

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