and thereby enhance its activity.[6,7] On the basis of this *in vivo* mechanism of storage, controlled release of heparin-binding growth factors has been described from heparin-carrying polystyrene-bound collagen substrata,[8] alginate gels containing heparin,[9] photo-crosslinked heparin hydrogel,[10] and chitosan/heparin hydrogels.[11]

Chitin is a linear homopolymer of 1, 4β-linked *N*-acetyl-D-glucosamine, and chitosan is a partially deacetylated chitin. Chitin and chitosan have been proposed for use as biomaterials with a range of biomedical and industrial applications because of their biocompatibility.[12] We previously reported the preparation of a novel water-soluble chitosan by introduction of lactose moieties.[13–15] The material obtained is a viscous solution and is easily gelled upon mixing with heparin[11] solution, resulting in an injectable hydrogel.

Fucoidans are vegetal fucose-containing polysaccharides extracted from brown algae. The contents of fucose, uronic acid, and sulfate in fucoidan were determined using extracted and purified fucoidan.[16] Fucoidan is known to bind heparin-binding growth factors such as FGF-1,[17] and enhance FGF-2 activity and FGF-2-induced tube formation of endothelial cells.[18]

In this study, we used fucoidan as a heparinoid to prepare an injectable chitosan/fucoidan micro complex-hydrogel. The purpose of the present study was to evaluate the chitosan/fucoidan micro complex-hydrogel as a carrier-material for controlled release of FGF-2, *in vitro* and *in vivo*. In addition, we examined the *in vivo* degradability of the hydrogel, as well as the effect of FGF-2-containing chitosan/fucoidan micro complex-hydrogel on vascularization and fibrous tissue formation. We report that chitosan/fucoidan micro complex-hydrogel has high affinity for FGF-2 molecules and is able to protect FGF-2 from inactivation.

MATERIALS AND METHODS

Preparation of fucoidan, water-soluble chitosan molecules (CH-LA), and chitosan/fucoidan micro complex-hydrogel

Fucoidan from *Kjellmaniella crassifolia* (Gagome-kombu) was prepared as described previously[16] with some minor modifications. Briefly, the Gagome-kombu used in this study was harvested from Hakodate, Hokaido, Japan. The collected seaweed was air-dried, and powdered in a blender. The powder was washed with 0.1*M* acetic acid in 70% ethanol, and extracted with 1.3 g/L $CaCl_2 \cdot 2H_2O$ in boiling water for 30 min. The supernatant was precipitated with 2 volumes of ethanol. The precipitate was dissolved with 0.9 wt % NaCl and 10 m*M* EDTA·2Na and then filtrated. Fucoidan was precipitated again with ethanol and dissolved with distilled water.

Water-soluble chitosan molecules (CH-LA; Yaizu Suisankagaku Industry, Shizuoka, Japan) were prepared as previously reported.[11] About 2% of the amino groups in the chitosan are substituted with lactobionic acid for dissolution in water at neutral pH-values.

Fucoidan aqueous solution (0.79 mL, 2.1 wt % v/v) was added to chitosan aqueous solution (0.21 mL, 4 wt % v/v) and mixed thoroughly with an ultrasonic mixer (Astrason; Misonix, NY) for 1 min. To minimize the production of large aggregation, the chitosan and fucoidan were mixed in a ratio of 1:2 (wt). The chitosan/fucoidan micro complex-hydrogel was then washed twice with phosphate-buffered saline (PBS) to remove unreactants, and brought to 1.0 mL with PBS (Fig. 1).

Assay of FGF-2 binding to chitosan/fucoidan micro complex-hydrogel

Chitosan/fucoidan micro complex-hydrogels were rinsed 3 times with PBS, and the hydrogel was suspended in PBS and a known concentration of ^{125}I-FGF-2 (NEX268, PerkinElmer Life Sci, MA) + FGF-2 (Fiblast; Kaken Pharmaceutical Corp., Tokyo, Japan), and bovine serum albumin (BSA; Wako Pure Chemical Industries, Osaka, Japan) were added to initiate binding. Specific binding was determined in the presence of 1 mg/mL of BSA. The binding reaction was continued for 30 min at room temperature. The radioactivity of ^{125}I-FGF-2 bound to the hydrogel was determined using a scintillation counter (AKC-2000; Aloka, Tokyo, Japan). The dissociation constant (K_d value) for specific binding of FGF-2 to the micro complex hydrogel was quantified using radioligand binding followed by Scatchard analysis.[19]

HMVEC culture and protection of FGF-2 from inactivation by chitosan/fucoidan micro complex-hydrogels

Human dermal micro vascular endothelial cells (HMVECs; Takara Biochemical Corp., Ohtsu, Japan) were cultured in Medium-199 (Life Technologies Oriental Corp., Tokyo, Japan) supplemented with 10 wt % heat-inactivated FBS and antibiotics (100 U/mL penicillin G and 100 μg/mL streptomycin). The cells used were all between the fourth and eighth cell cycle passage.

To study the efficacy of the chitosan/fucoidan micro complex-hydrogels in inhibiting FGF-2 inactivation with various times of incubation, 1 mg of the chitosan/fucoidan micro complex-hydrogels, CH-LA, or fucoidan was added to 1 mL of FGF-2 (50 μg/mL) in PBS and thoroughly mixed. These stock solutions were directly incubated at 37°C for the indicated number of days. For heat treatment of FGF-2, 0.2 mL of each stock solution was heated at 37, 44, 51, 58, 65, or 72°C for 30 min. To study the effect of trypsinization of FGF-2, 0.1 mL of 0.05% trypsin-EDTA solution (Sigma, St. Louis, MO) was added to 0.2 mL of each chilled stock solution and incubated at 37°C for the indicated period of time. After incubation,

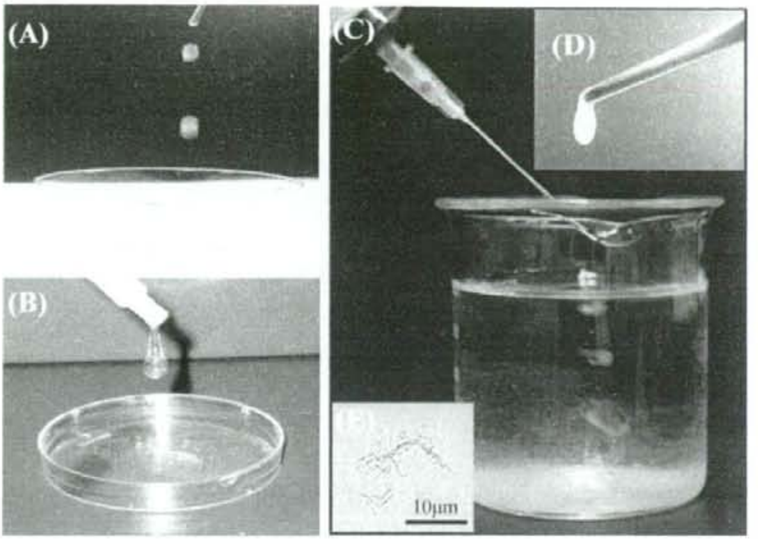

Figure 1. Fucoidan aqueous solution (A) and chitosan solution with low viscosity (B) were mixed to produce chitosan/fucoidan micro complex-hydrogel (C) under the conditions described in Materials and Methods. The chitosan/fucoidan micro complex-hydrogel is able to pass easily through a needle (C) and can be picked up with forceps (D). The chitosan/fucoidan micro complex-hydrogel consists of an insoluble fiber complex which is approximately 10–20 μm in dimension (E).

0.2 mL of heat-inactivated FBS was added to each trypsin-stock solution to stop the trypsinization reaction.

Inactivated FGF-2 solutions were diluted with culture medium for HMVEC at indicated concentrations, and used for HMVEC culture. HMVECs were seeded at an initial density of 3000 cells/well in 96-well tissue culture plates and grown for 3 days in 200 μL of one of the prepared media. After incubation, the medium was replaced with 100 μL of fresh medium including 10 μL of WST-1 reagent (Cell Counting Kit, Dojindo, Kumamoto, Japan) and incubated at 37°C for 1 h. The optical density (OD) of each well was read at 450 nm in an Immuno Mini plate reader (Nunc InterMed Japan, Tokyo, Japan).

Endothelial cell growth assay *in vitro*

HMVECs (5×10^3/well) were seeded on 96-well tissue culture plates in 100 μL of culture medium, (1) the indicated concentration of either native heparin, CH-LA, fucoidan, or chitosan/fucoidan micro complex-hydrogels, and (2) the indicated concentration of FGF-2, and grown for 3 days. After incubation, the OD was read at 450 nm as described earlier.

Assessment of neovascularization induced by FGF-2-containing chitosan/fucoidan micro complex-hydrogels

Hundred microliters of the FGF-2 (50 μg/mL) -containing chitosan/fucoidan micro complex-hydrogel was injected into the subcutis on the back of the mice (C57BL/6J male mice; CLEA Japan, Tokyo, Japan). At the indicated day after hydrogel injection, the tissues dissected to evaluate neovascularization from mice injected hydrogel were fixed with 10% formaldehyde, and then subjected to the standard histological processing for hematoxylin-eosin (H&E) staining.[10] In each sections, an area randomly chosen per injection site was photographed (microscopic fields, 100×) and the number of vessels containing erythrocytes per microphotograph was counted. Animal experiments were carried out according to the protocol approved by *the Animal Experimentation Committee of the National Defense Medical College*.

Statistical analysis

Statistical analysis was carried out using the unpaired Student's *t*-test with mean and standard error determinations for each experimental group.

RESULTS

Assay of FGF-2 binding to chitosan/fucoidan micro complex-hydrogel

In the radioligand binding inhibition assay, 90–80% of ^{125}I-FGF-2 bound to the chitosan/fucoidan micro complex hydrogel, and binding was not reduced by adding either BSA up to 2 mg/mL or FGF-2 up to

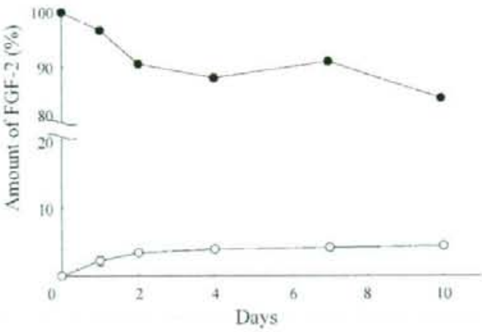

Figure 2. Profile of release of FGF-2 from FGF-2-containing chitosan/fucoidan micro complex-hydrogel. Amounts of FGF-2 initially incorporated into the chitosan/fucoidan micro complex-hydrogel were considered as 100%. The amount of FGF-2 molecules released into DMEM at 37°C (○) and the amount of FGF-2 molecules retained in chitosan/fucoidan micro complex-hydrogel (●) were quantified, as described in Materials and Methods. Each data point represents the mean of triplicate determinations.

200 μg/mL. Therefore, the binding of FGF-2 to the chitosan/fucoidan micro complex-hydrogel appeared mostly to be specific (data not shown).

When ^{125}I-FGF-2 in the chitosan/fucoidan micro complex-hydrogel (50 μg/mL) was incubated with the culture medium at 37°C, about 5% of the incorporated FGF-2 molecules were released from the hydrogels within 2 days with no substantial release thereafter (Fig. 2). It may be explained in terms of this molecular diffusion, because the hydrogels in an *in vitro* condition would not be attacked by enzymatic digestion. The present FGF-2 immobilization conditions in the hydrogel were sufficient to complete complex formation between FGF-2 molecules and fucoidan, resulting in little diffusion of uncomplexed FGF-2.

Saturation curves and Scatchard plots for the binding of ^{125}I-FGF-2 to the chitosan/fucoidan micro complex-hydrogel yielded a dissociation constant of $K_d = 5.4 \times 10^{-9}M$ (Fig. 3). Heparin is known to have high affinity for FGF-2 ($K_d = 8.6 \times 10^{-9}M$)[19] and to enhance and protect FGF-2 activity. Since the binding of FGF-2 to chitosan hydrogel is much lower than that to fucoidan and heparin ($K_d = 6.12 \times 10^{-7}M$),[19] FGF-2 is able to bind to the chitosan/fucoidan micro complex-hydrogel with high affinity through interaction between FGF-2 and fucoidan molecules.

Chitosan/fucoidan micro complex-hydrogel protects FGF-2 from inactivation

When FGF-2 in PBS was incubated at 37°C for 1 day or more, the mitogenic activity of FGF-2 was to a large extent lost (<90%), whereas no decrease in mitogenic activity of FGF-2 was observed in the presence of either 1 mg/mL fucoidan or chitosan/fucoidan micro complex-hydrogel over at least 7 days (Fig. 4). The biological half-lives of 10 ng/mL of FGF-2 in the presence of chitosan/fucoidan micro complex-hydrogel, fucoidan, CH-LA, and control were approximately >7, >7, 3, and <1 days, respectively.

Heparin protects FGF-2 from inactivation by heat and proteolysis.[20] To determine whether the chitosan/fucoidan micro complex-hydrogel could protect FGF-2 against heat inactivation, FGF-2 was heated in the presence of chitosan/fucoidan micro complex-hydrogel (1 mg/mL). Exposure of FGF-2 to temperatures above at over 51°C in the absence of CH-LA, fucoidan, and chitosan/fucoidan micro complex-hydrogel resulted in a greater 95% inactivation, whereas CH-LA almost completely protected FGF-2 activity at temperatures up to 58°C and partially protected it at 65°C (Fig. 5). On the other hand, fucoidan and chitosan/fucoidan micro complex-

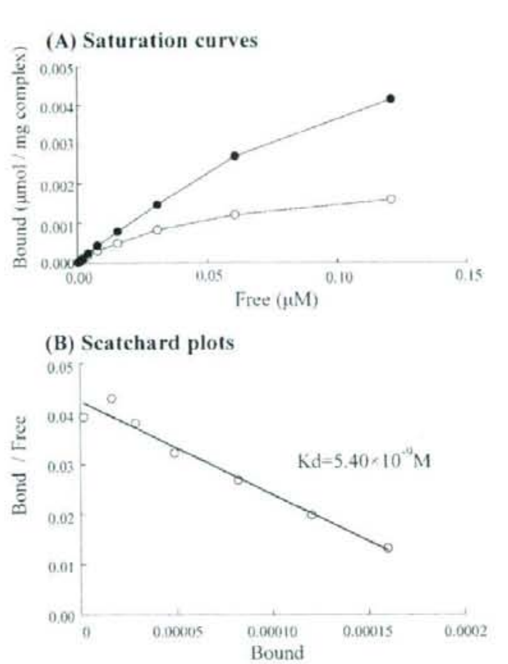

Figure 3. Saturation curves (A) and Scatchard plots (B) for the binding of ^{125}I-FGF-2 to chitosan/fucoidan micro complex-hydrogel. In the saturation curves, nonspecific binding (●) was obtained by coating the chitosan/fucoidan micro complex-hydrogel complexed with 0.1% BSA, and the specific binding (○) was calculated by subtraction of nonspecific binding from total binding. Each data point in saturation curves represents the mean of triplicate determinations.

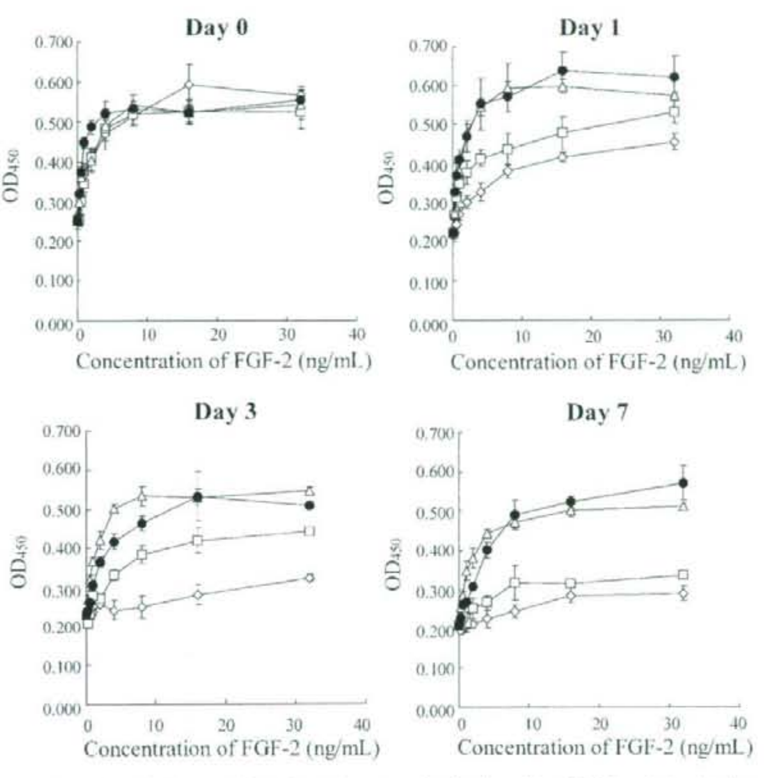

Figure 4. The protective effect of chitosan/fucoidan micro complex-hydrogel for FGF-2 activity. Stock solutions (50 μg/mL FGF-2 in chitosan/fucoidan micro complex-hydrogel (●), CH-LA (□), fucoidan (△), and PBS (◇) were incubated at 37°C for 0, 1, 3, and 7 days. FGF-2 in stock solution was diluted to the indicated concentration with culture medium. HMVECs were cultured for 3 days using one of the prepared media. Results are the mean ± SD of four determinations.

hydrogel exhibited greater protection of FGF-2 activity up to 65°C.

The CH-LA, fucoidan, and chitosan/fucoidan micro complex-hydrogel were able to protect against inactivation of FGF-2 by trypsin, while PBS could not (Fig. 6). In the presence of trypsin, the biological half-lives of 10 ng/mL of FGF-2 in the presence of CH-LA, fucoidan, and chitosan/fucoidan micro complex-hydrogel were about 75, 80, and >120 min, respectively.

Endothelial cell growth assay *in vitro*

HMVECs were not able to grow in medium in the presence of 10% FBS without addition of a specific growth factor. When FGF-2 (5 ng/mL) was added to the culture medium, the rate of growth of HMVECs increased 1.7-fold compared to the control. The horizontal lines in Figure 7(A,B) show cell growth in the medium containing 10% FBS in the absence of exogenous growth factors. While addition of low concentrations (below 8 μg/mL) of heparin, fucoidan, and chitosan/fucoidan micro complex-hydrogel to the medium did not influence the growth of HMVECs in the presence of 10% FBS and 5 ng/mL FGF-2, high concentrations (above 16 μg/mL) significantly inhibited cell growth in dose-dependent fashion [Fig. 7(A)]. On the other hand, up to 256 μg/mL CH-LA did not inhibit cell growth.

Although the concentration of FGF-2 required for half activity (CA_{50}) without heparin, CH-LA, fucoidan, and chitosan/fucoidan micro complex-hydrogel was about 2 ng/mL, in the presence of either heparin, CH-LA, fucoidan, or chitosan/fucoidan micro complex-hydrogel, CA_{50} values were approximately <0.25, <0.5, <0.25, and <0.25 ng/mL [Fig. 7(B)]. Thus, fucoidan and chitosan/fucoidan micro complex-hydrogel as well as heparin enhanced FGF-2 activity.

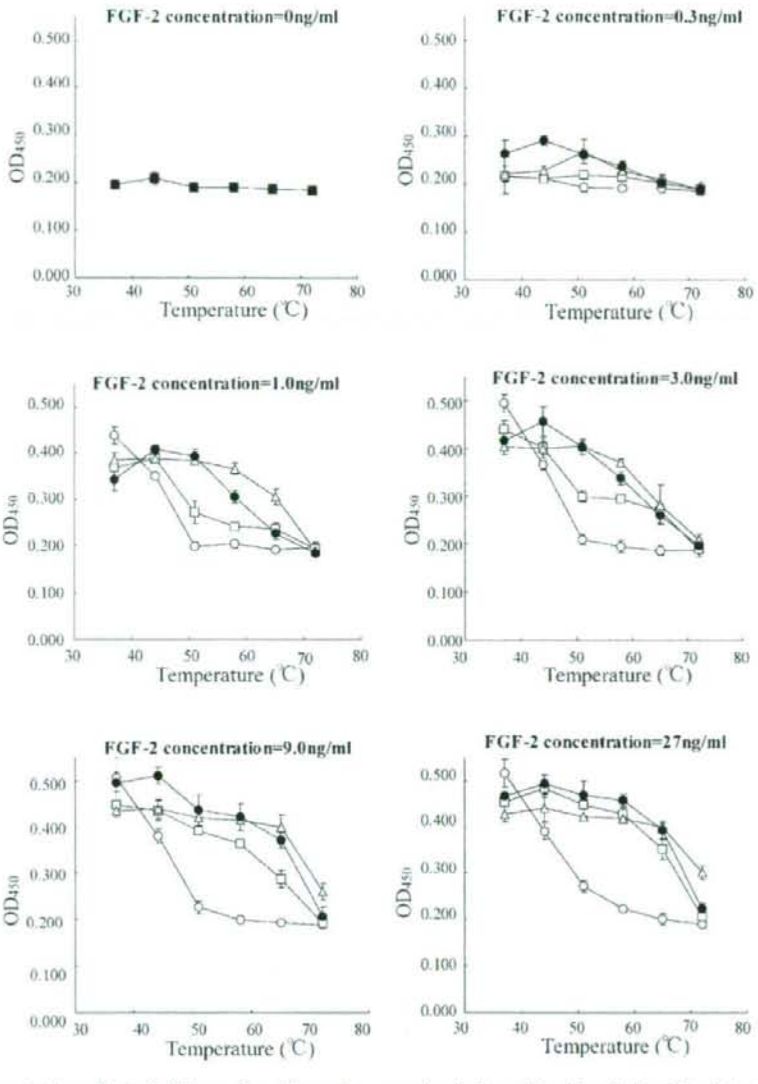

Figure 5. The protective effect of chitosan/fucoidan micro complex-hydrogel for bioactivity of heat-treated FGF-2. Stock solutions (50 μg/mL FGF-2 in chitosan/fucoidan micro complex-hydrogel (●), CH-LA (□), fucoidan (△), and PBS (○)) were incubated at 37, 44, 51, 58, 65, and 72°C for 30 min. FGF-2 in stock solution was diluted to the indicated concentration with the culture medium. HMVECs were cultured for 3 days using one of the prepared media. Results are the mean ± SD of four determinations.

Histological observations of vascularization *in vivo*

To confirm the effects of FGF-2-containing chitosan/fucoidan micro complex-hydrogel on vascularization and fibrous tissue formation in subcuits of the back in mice, histological observations were performed with H&E staining. On 1 week, considerable amounts of injected FGF-2-containing chitosan/fucoidan micro complex-hydrogels and chitosan/fucoidan micro complex-hydrogels remained with numerous neutrophils migrating into the gel (Fig. 8). However, until 4 weeks, there was visual evidence of a complete biodegradation of hydrogels at the injection sites (data not shown). The duration of

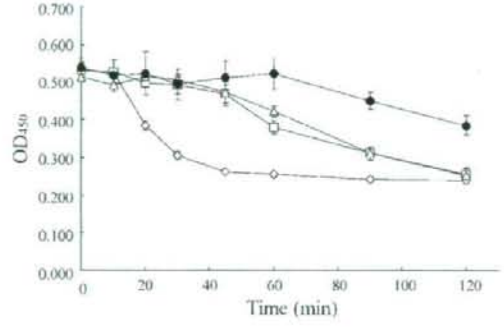

Figure 6. The protective effect of chitosan/fucoidan micro complex-hydrogel for bioactivity of trypsin-treated FGF-2. Stock solutions (50 μg/mL FGF-2 in in chitosan/fucoidan micro complex-hydrogel (●), CH-LA (□), fucoidan (△), and PBS (◇) were treated with trypsin at 37°C for the indicated periods of time. After trypsinization, proteolysis was stopped by adding FBS, and the inactivated FGF-2 in stock solution was diluted to 10 ng/mL with culture medium. HMVECs were cultured for 3 days using one of the prepared media. Results are the mean ± SD of four determinations.

biodegradation of the hydrogel was roughly estimated to be about 2 weeks. The areas in which FGF-2-containing chitosan/fucoidan micro complex-hydrogels remainind in the tissue removed at 1 week after injection were covered by glossy smooth pleura (Fig. 8). The FGF-2-containing chitosan/fucoidan micro complex-hydrogels were also encapsulated by fibrous tissue and covered with regenerated thick pleura, this continued to be observed until 4 weeks. These fibrous tissues were not observed after injections of FGF-2 alone or chitosan/fucoidan micro complex-hydrogels.

The number of capillaries per microphotograph (100×) in tissues near the sites of injection at weeks 1, 2, 3, and 4 were determined in Figure 9. Numerous mature vessels containing erythrocytes were already observed around the injected FGF-2-containing chitosan/fucoidan micro complex-hydrogels from week 1 onward (Fig. 8). Although neovascularization was not observed on day 1 to day 2, it was observed from day 3 (data not shown).

DISCUSSION

We previously reported a photo-crosslinked chitosan hydrogel with controlled release of various growth factors, serving as a novel carrier of growth factors and inducing neovascularization and granulation tissue formation *in vivo*.[10] Furthermore, it was demonstrated that FGF-2 molecules incorporated into the photo-crosslinked chitosan hydrogel were gradually released upon biodegradation of the hydrogel, and that FGF-2-containing photo-crosslinked chitosan hydrogels markedly improved wound healing in healing-impaired *db/db* mice,[15] although the photo-crosslinked chitosan hydrogel was not injectable. The chitosan molecule has low affinity for FGF-2 (K_d of $6.12 \times 10^{-7}M$), at 1 mg/mL prolongs the biological half-life of FGF-2, and chitosan protects FGF-2 molecules from heat and proteolytic inactivation.[19]

FGF-2 is bind to heparin with high affinity (K_d of $8.6 \times 10^{-9}M$).[19] Polysaccharides also prolong the biological half-life of FGF-2, as well as protect it from heat, acid, and proteolytic inactivation with

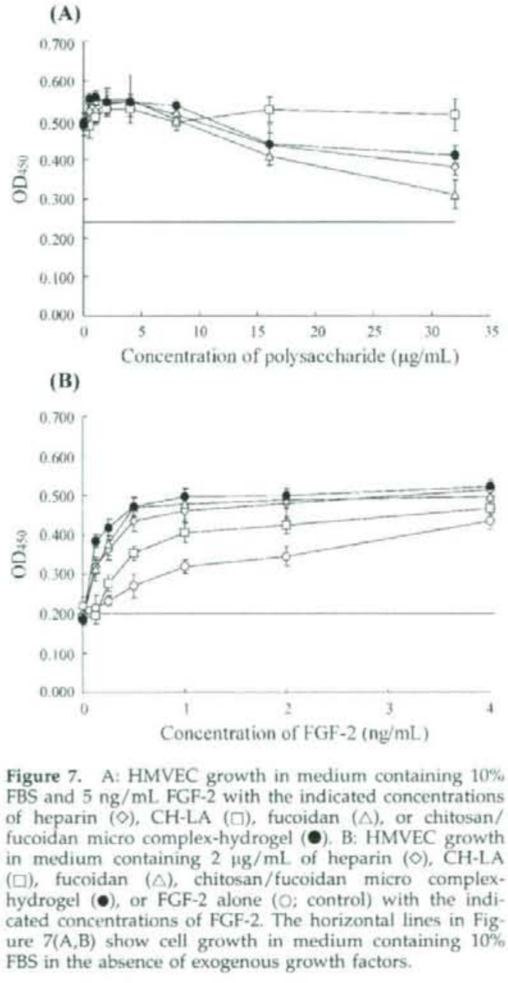

Figure 7. A: HMVEC growth in medium containing 10% FBS and 5 ng/mL FGF-2 with the indicated concentrations of heparin (◇), CH-LA (□), fucoidan (△), or chitosan/fucoidan micro complex-hydrogel (●). B: HMVEC growth in medium containing 2 μg/mL of heparin (◇), CH-LA (□), fucoidan (△), chitosan/fucoidan micro complex-hydrogel (●), or FGF-2 alone (○; control) with the indicated concentrations of FGF-2. The horizontal lines in Figure 7(A,B) show cell growth in medium containing 10% FBS in the absence of exogenous growth factors.

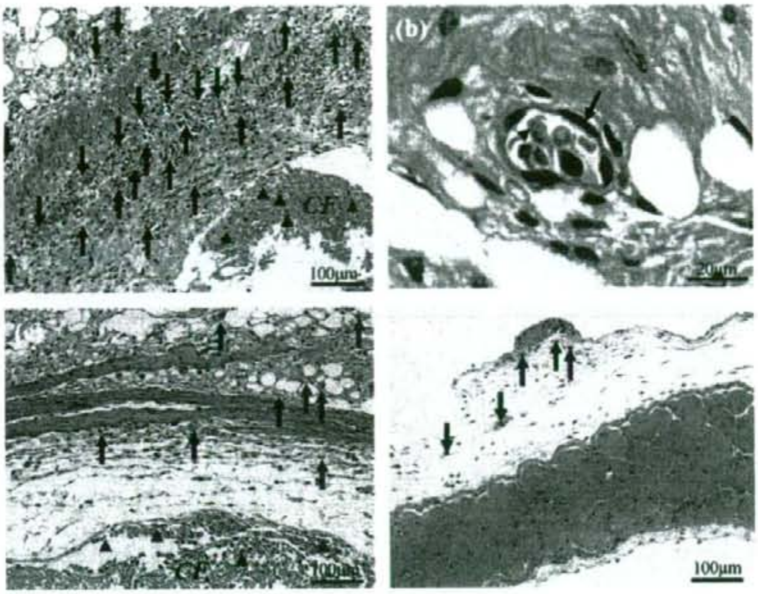

Figure 8. Histological examination of sites injected with FGF-2-containing chitosan/fucoidan micro complex-hydrogel [(a) and (b)], chitosan/fucoidan micro complex-hydrogel alone (c), or FGF-2 alone (d) at 1 week after injection. Black arrows indicate vessels in Figures (a), (c), and (d). In Figure (b), magnified from the sample injected with FGF-2-containing chitosan/fucoidan micro complex-hydrogel (original magnification ×400), vessels containing endothelial cells (indicated by black arrows) and erythrocytes (by arrowheads) are observed. Considerable amounts of injected chitosan/fucoidan micro-hydrogel [CF in (a) and (b)] remained, with infiltration by numerous neutrophils [indicated by arrowheads in (a) and (c)].

concentrations up to 10 μg/mL.[20] Similarly, the chitosan/fucoidan micro complex-hydrogel has high affinity for FGF-2 ($K_d = 5.4 \times 10^{-9}M$). The interaction

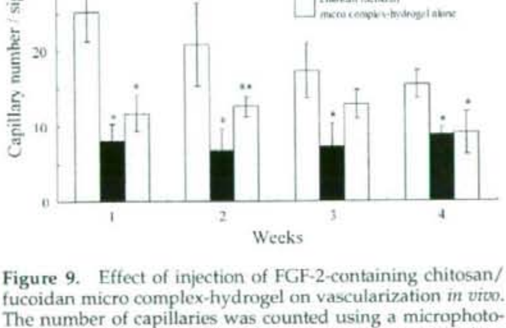

Figure 9. Effect of injection of FGF-2-containing chitosan/fucoidan micro complex-hydrogel on vascularization *in vivo*. The number of capillaries was counted using a microphotograph (Fig. 8) of each section ($n = 6$) exhibiting the highest mature capillary density. All values are means ± SD. For comparison of experimental and control groups, the unpaired *t*-test was used, with findings of $p < 0.001$ marked with an asterisk and of $p < 0.01$ with a double asterisk.

of FGF-2 with the chitosan/fucoidan micro complex-hydrogel also substantially prolonged the biological half-life of FGF-2. Protection of FGF-2 against heat inactivation and trypsin degradation by the chitosan/fucoidan micro complex-hydrogel was effective at concentrations up to less than 10 μg/mL (data not shown). The results demonstrated that FGF-2 molecules are bound and stabilized in the chitosan/fucoidan micro complex-hydrogel, and that the FGF-2 molecules incorporated in the chitosan/fucoidan micro complex-hydrogel are gradually released upon biodegradation of the hydrogel *in vivo*. Although we did not study the detailed degradation-process of this hydrogel, it may be degraded by an enzymatic digestion of endoglycosidase from infiltrated leukocytes and phagocytosed by the infiltrated leukocytes.[15] When the FGF-2-containing chitosan/fucoidan micro complex-hydrogel was subcutaneously injected into the backs of mice, neovascularization was induced near the injection site after 3 days and was reached a maximum at 1 week, after which a slight decrease in neovascularization rate occurred. No significant vascularization was observed after the injection of FGF-2 alone or the chitosan/fucoidan micro complex-hydrogel. These findings suggest that injection of FGF-2 without a hydrogel carrier causes excessively rapid diffusion of FGF-2 molecules from the injected site to induce any vascularization effect.

It is recognized in polymer chemistry that positively and negatively charged polymers interact ionically. Basic chitosan molecules complexed with acidic molecules (fucoidan) form a hydrogel through ionic interactions. It seems likely that polypeptides such as FGF-2, once ionically complexed with chitosan or fucoidan, are not released from the hydrogel. Since the chitosan/fucoidan micro complex-hydrogel is biodegradable *in vivo*, incorporating polypeptides in the hydrogel will provide an excellent controlled-release system over a 2-week period.

Although heparin is clinically used as an antithrombotic agent, the dose is limited by its strong intrinsic anticoagulant ability, with the potential for severe bleeding complications. Fucoidan, a heparinoid, lacks a specific pentasaccharide structure that interacts with antithrombin III,[7] and its anticoagulant activity is much lower than that of native heparin.[21] Fucoidan is known to bind heparin-binding growth factors such as FGF-1,[17,22] and enhance FGF-2 activity *in vitro*.[18,22] Furthermore, we showed that fucoidan has high affinity for FGF-2 like heparin, and protects FGF-2 from inactivation. Therefore, fucoidan was used to prepare the chitosan/fucoidan micro complex-hydrogel in this study. In fact, bleeding complications were not observed at the injected sites. Our findings suggest that FGF-2-containing chitosan/fucoidan micro complex-hydrogel may be a promising new biomaterial for induction of vascularization and fibrous tissue formation in ischemic limbs.

References

1. Gospodarowicz D. Fibroblast growth factor and its involvement in developmental processes. Curr Top Dev Biol 1990;24:57–93.
2. Tabata Y, Ikada Y. Vascularization effect of basic fibroblast growth factor released from gelatin hydrogels with different biodegradabilities. Biomaterials 1999;20:2169–2175.
3. Ishihara M, Ono K. Structure and function of heparin and heparan sulfate: Heparinoid library and modification of FGF-activities. Trends Glycosci Glycotechnol 1998;10:223–233.
4. Slack JM, Darlington BG, Heath JK, Godsave SF. Mesoderm induction in early *Xenopus* embryos by heparin binding growth factors. Nature 1987;326:197–200.
5. Canalis E, Centrella M, McCarthy T. Effects of basic fibroblast growth factor on bone formation *in vitro*. J Clin Invest 1988; 81:1572–1577.
6. Ishihara M. Biosynthesis, structure, and biological activity of basic FGF binding domains of heparan sulfate. Trends Glycosci Glycotechnol 1993;5:343–354.
7. Lindahl U, Lidholt K, Spillmann D, Kjellen L. More to "heparin" than anti-coagulation. Thromb Res 1994;75:1–32.
8. Ishihara M, Sato M, Hattori H, Saito Y, Yura H, Ono K, Masuoka K, Kikuchi M, Fujikawa K, Kurita A. Heparin-carrying polystyrene (HCPS)-bound collagen substratum to immobilize heparin-binding growth factors and to enhance cellular growth. J Biomed Mater Res 2001;56:536–544.
9. Tanihara M, Suzuki Y, Yamamoto E, Noguchi A, Mizushima Y. Sustained release of basic fibroblast growth factor and angiogenesis in a novel covalently crosslinked gel of heparin and alginate. J Biomed Mater Res 2001;56:216–222.
10. Ishihara M, Obara K, Ishizuka T, Fujita M, Sato M, Masuoka K, Saito Y, Yura H, Matsui T, Hattori H, Kikuchi M, Kurita A. Controlled release of fibroblast growth factors and heparin from photocrosslinked chitosan hydrogels and subsequent effect on *in vivo* vascularization. J Biomed Mater Res A 2003; 64:551–559.
11. Fujita M, Ishihara M, Shimizu M, Obara K, Ishizuka T, Saito Y, Yura H, Morimoto Y, Takase B, Matsui T, Kikuchi M, Maehara T. Vascularization *in vivo* caused by the controlled release of fibroblast growth factor-2 from an injectable chitosan/non-anticoagulant heparin hydrogel. Biomaterials 2004; 25:699–706.
12. Shigemasa Y, Minami S. Application of chitin and chitosan for biomaterials. Biotechnol Genet Eng Rev 1995;13:383–420.
13. Ono K, Saito Y, Yura H, Ishikawa K, Kurita A, Ishihara M. Photocrosslinkable chitosan as a biological adhesive. J Biomed Mater Res 2000;49:289–295.
14. Ishihara M, Nakanishi K, Ono K, Sato M, Saito Y, Yura H, Matsui T, Hattori H, Uenoyama M, Kikuchi M, Kurita A. Photocrosslinkable chitosan as a dressing for wound occlusion and accerelator in healing process. Biomaterials 2002; 23:833–840.
15. Obara K, Ishihara M, Ishizuka T, Fujita M, Ozeki Y, Maehara T, Saito Y, Yura H, Matsui T, Hattori H, Kikuchi M, Kurita A. Photocrosslinkable chitosan hydrogel containing fibroblast growth factor-2 stimulates wound healing in healing-impaired *db/db* mice. Biomaterials 2003;24:3437–3444.
16. Nishide E, Anzai H, Uchida N. A comparative investigation on the contents of fucose-containing polysaccharides from various Japanese brown algae. Nippon Suisan Gakkai Shi 1987;53:1083–1088.
17. Belford DA, Hendry IA, Parish CR. Investigation of the ability of several naturally occurring and synthetic polyanions to bind to and potentiate the biological activity of acidic fibroblast growth factor. J Cell Physiol 1993;157:184–189.
18. Luyt CE, Meddahi-Pelle A, Ho-Tin-Noe B, Colliec-Jouault S, Guezennec J, Louedec L, Prats H, Jacob M-P, Osborne-Pellegrin M, Letourneur D, Michel J-B. Low-molecular-weight fucoidan promotes therapeutic revascularization in a rat model of critical hindlimb ischemia. J Pharmacol Exp Ther 2003;305:24–30.
19. Masuoka K, Ishihara M, Asazuma T, Hattori H, Matsui T, Takase B, Kanatani Y, Fujita M, Saito Y, Yura H, Fujikawa K, Nemoto K. The interaction of chitosan with fibroblast growth factor-2 and its protection from inactivation. Biomaterials 2005;26:3277–3284.
20. Gospodarowicz D, Cheng J. Heparin protects basic and acidic FGF from inactivation. J Cell Physiol 1986;128:475–484.
21. Millet J, Jouault SC, Mauray S, Theveniaux J, Sternberg C, Boisson-Vidal C, Fischer AM. Antithrombotic and anticoagulant activities of a low molecular weight fucoidan. Thromb Haemost 1999;81:391–395.
22. Giraux J, Matou S, Bros A, Tapon-bretaudiere J, Letourneur D, Fischer A. Modulation of human endothelial cell proliferation and migration by fucoidan and heparin. Eur J Cell Biol 1998;77:352–359.

Expansion and Characterization of Adipose Tissue-derived Stromal Cells Cultured With Low Serum Medium

Hidemi Hattori,[1] Yashiro Nogami,[2] Tomohiro Tanaka,[3] Yoshiko Amano,[1] Koichi Fukuda,[3] Satoko Kishimoto,[1] Yasuhiro Kanatani,[1] Shingo Nakamura,[2] Bonpei Takase,[1] Masayuki Ishihara[1]

[1] Research Institute, National Defense Medical College, Tokorozawa, Saitama 359-8513, Japan

[2] Department of Surgery, National Defense Medical College, Tokorozawa, Saitama 359-8513, Japan

[3] Center for Laboratory Animal Science, National Defense Medical College, Tokorozawa, Saitama 359-8513, Japan

Received 12 July 2007; revised 9 January 2008; accepted 4 February 2008
Published online 21 May 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.b.31101

Abstract: Adipose tissue contains a population of cells that have extensive self-renewal capacity and the ability to differentiate along multiple lineages. In addition, adipose tissue-derived stromal cells (ATSCs) are able to differentiate into various cell types that may be useful for autologous cell transplantation for defects of bone, cartilage, adipose, and tendon, etc. Most protocols for *in vitro* cultures of ATSCs include fetal bovine serum (FBS) as a nutritional supplement. However, in some cell cultures, it involves multiple doses of FBS, which raises a concern over possible infections as well as immunological reactions that are caused by medium-derived FBS proteins, sialic acid, etc. In this study, we were able to expand mouse ATSCs using low mouse serum media containing collagen type I, heparin-carrying polystyrene, and fibroblast growth factor (FGF)-2. These expanded mouse ATSCs maintained their multilineage potential for differentiation into adipocytes, osteoblasts, and chondrocytes. Therefore, this method, which uses autologous cells and low serum media, may be able to be utilized for clinical cell therapies. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 87B: 229–236, 2008

Keywords: adipose tissue stromal cells; differentiation; heparin-carrying polystyrene; cell growth; low serum

INTRODUCTION

Adipose tissue is a highly complex organ consisting of mature adipocytes, preadipocytes, fibroblasts, vascular smooth muscle cells, endothelial cells, monocytes, macrophages, and lymphocytes, among others. In addition, adipose tissue-derived stromal cells (ATSCs) have the ability to differentiate into a number of different cell types.[1,2] If there was a source of autologous multipotential stem cells, this would be of great benefit to cell-based therapies such as tissue engineering. Adipose tissue compartments are a rich source that is very useful when preparing autologous progenitor cells for cell-based therapies. Moreover, to obtain these cells, fat suction in humans usually is performed under local and epidural anesthesia, the multipotent characteristics of ATSCs, as well as their abundance in the human body, make these cells a potential source in wound repair and tissue engineering applications. However, in some cases for patients who require cell-based therapies to repair damaged tissues, there are not enough cells that can be prepared, so these cells need to be cultured for expansion and use. Additionally, for specific clinical applications, cell cultures may be the only way to expand the progenitor cells that are required for the applications.

Recently, the persistence of xenogeneic proteins in human mesenchymal stem cells or bone marrow stromal cells (MSCs)[3,4] that were expanded with fetal bovine serum (FBS) have been examined extensively.[5] Results from the study indicated that after intravenous administration of autologous rat MSCs expanded with FBS, humoral immune responses against FBS proteins were observed in the recipients. Moreover, it has been reported that currently available lines of human embryonic stem (ES) cells have been contaminated with a non-human molecule that can compromise their potential therapeutic use in human subjects. These ES cells were found to be contaminated with substantial amounts of sialic acid.[6] Most culture methods used to grow human ES cells require animal-derived materials, including connective tissue cells (feeder layers) from mice and FBS.

Correspondence to: M. Ishihara (e-mail: ishihara@ndmc.ac.jp)

Thus, problems may surface in patients undergoing autogenous cell-based therapies if a serum other than an autologous serum is used during the culturing of the cells.

Heparin and heparan sulfate (HS) are members of the glycosaminoglycans and are normally present as proteoglycans (PGs), for which a number of heparin/HS-chains are covalently attached to a core protein. Aside from its well-known anti-coagulant action, heparin molecules have been found to be associated with growth factors and cytokines in various biological processes, as well as being involved in cell adhesion, recognition, migration, and regulation of various enzymatic activities.[7,8] Growth factors such as fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF) are especially immobilized on the extracellular matrix via binding to heparan sulfate proteoglycan (HSPG).

Previously, we reported that periodate-treated, nonanticoagulant heparin-carrying polystyrene (HCPS) has been found to be a synthetic glyco-conjugate that is soluble in water and acts as a HSPG mimic. HCPS exhibits significantly reduced anti-coagulant activity and enhanced abilities to interact with various heparin-binding growth factors, such as FGF-2, $VEGF_{165}$, and HGF, which are known to stimulate angiogenesis. In fact, it has been found to be effective in the growth of various cells when culture plates are covered with various HCPS-immobilized growth factors.[9–11]

An ATSCs culturing method with a low serum concentration (1%) would be very useful for tissue engineering, as a large quantity of blood would not have to be collected, thus making the process less invasive for the patient. Furthermore, such a procedure would make it possible to use autologous serum for the culturing of the ATSCs. The purpose of this study was to establish a method for expanding ATSCs under low mouse serum (MS) conditions (1%) via the use of a plate covered with HCPS-immobilized FGF-2.

MATERIALS AND METHODS

Preparation of Mouse ATSCs

Primary ATSCs were prepared from inguinal adipose tissue of 8-week-old ICR mice (Japan SLC, Shizuoka, Japan). The adipose tissue was washed extensively with Dulbecco's modified Eagle's medium (DMEM) and digested for 0.5, 1, 2, or 4 h at 37°C with 0.1% collagenase type I (Wako Pure Chemical Industries, Osaka, Japan). The cells were suspended in the medium and treated with a 100-μm nylon mesh (Becton Dickinson, NJ). The cells were resuspended in DMEM containing 10% MS and antibiotics (100 U/mL penicillin G and 100 μg/mL streptomycin) (control medium), and then centrifuged at 3000 rpm for 10 min. The cells obtained were used as the ATSCs. Cell numbers in the ATSCs fractions were determined using a hemocytometer after a hemolytic reagent treatment that employed 160 mM NH_4Cl. The cell numbers in the adherent fractions were determined using a hemocytometer after trypsinization. On day 3, the adherent cells in the cultures were considered to be ready for further experiments.

Selection of Culture Media

ATSCs were plated at 5000 cells/well on 48-well suspension culture plates (well areas, 0.65 cm^2) using one of 4 types of basal media (Asahi Techno Glass Co., Tokyo, Japan). These media included modified minimum essential medium (MEM), DMEM, RPMI1640, and DMEM/Ham's nutrient mixture F12 (DMEM/F12). Each medium was supplemented with 1, 5, and 10% MS, or 1, 5, and 10% FBS and antibiotics (100 U/mL penicillin G and 100 μg/mL strptomycin). The cell numbers were determined using a hemocytometer after trypsinization.

Growth of ATSC on Heparin-Binding Growth Factor-Immobilized, HCPS-Bound Collagen Type I Substratum

The 48-well suspension culture plates were coated overnight at 4°C with 150 μL of 0.03 wt % collagen type I in acidic water (Koken Co., Tokyo, Japan). The collagen solutions were removed from the wells by suction, and then washed twice with PBS. HCPS was prepared using a previously reported method.[9–11] Collagen-coated plates were coated overnight at 4°C with 150 μL of 0.1 wt % HCPS solution. The HCPS solutions were subsequently removed from the wells by suction, and the plates were then washed twice with PBS. ATSCs were plated in DMEM/F12 containing 1% MS at an initial density of 5000 cells/well. The wells contained FGF-2, $VEGF_{165}$, or HGF (R&D Systems, MN). After 3 days, the cell numbers were determined using a hemocytometer after trypsinization.

Induction of Osteoblast, Adipocyte, and Chondrocyte Differentiation

Adipocyte, osteoblast, and chondrocyte differentiations were induced by placing the ATSCs in an adipogenic differentiation medium (Cambrex Bio Science Walkersville, MD), or in an osteogenic medium containing 150 ng/mL of bone morphogenetic proteins 2 (BMP-2) (R&D systems) and 5% FBS, or in chondrogenic medium using chondrogenic differentiation medium (Cambrex Bio Science Walkersville), respectively. For adipocyte differentiation, the medium was replaced with control medium containing 10 μg/mL of insulin on day 2. To induce mineralization of the osteogenic characterization, the cells were cultured with osteogenic medium containing 50 μM ascorbate-2-phosphate and 2 mM β-glycerophosphate. For the differentiation of the chondrocytes, the micromass pellets (2.5×10^5 cells) were cultured in chondrogenic differentiation medium. Under low-speed centrifugation, a dense mass of cells formed at the bottom of the conical centrifuge tube

(Becton Dickinson). Within 1 day, the cells consolidated to a single mass.

Characterization of Cell Properties

The adipocytes were stained with oil red O (Hokudo Co., Hokkaido, Japan). The amount of adiponectin secreted into the culture medium was determined with the mouse adiponectin Enzyme-Linked ImmunoSorbent Assay kit (Otsuka Pharmaceutical Co., Tokyo, Japan). Alkaline phosphatase (ALP) cytochemistry was carried out by using an ALP stain kit (Takara Bio, Shiga, Japan). ALP activity in the cell layer was measured as has been previously described.[12] Cell layers were sonicated in 50 m*M* Tris-HCl, 0.1% Triton X-100, pH 7.5. ALP activity in the lysate was measured at 37°C in a buffer containing 0.1*M* 2-amino-2-methyl-1-propanol (Sigma) and 2 m*M* $MgCl_2$, pH 10.5, for 30 min using *p*-nitrophenyl phosphate as the substrate. The ALP activity was expressed as micromoles of *p*-nitrophenol produced per min per mg of protein. The protein content was determined using a BCA protein assay kit (Pierce Biotechnology, IL). Detection of calcium depositions was carried out using a stock solution of 1% alizarin red S (Wako). Cartilage-specific anionic sulfated PGs were detected by toluidine blue metachromasia. After culturing for 21 days, the pellets were fixed with 4% paraformaldehyde and embedded in paraffin. Subsequently, 7-μm thick sections were cut and then stained with toluidine blue.

RESULTS

Collagenase Type I Treating Time for ATSCs Obtained From Adipose Tissue

The number of ATSCs obtained from ICR mice inguinal adipose tissue was examined to determine if any alterations occurred that were directly related to the changes in the collagenase type I treatment time. About 0.5 g of adipose tissue was obtained from the inguinal area of an 8-week-old ICR mouse. There was a time-dependent increase in the amount of the ATSCs obtained when the processing time with the collagenase type I was lengthened. However, there was little difference noted when the processing time was greater than 2 h (Figure 1). Therefore, we set the treatment time at 2 h for all of the experiments using adipose tissue with collagenase type I.

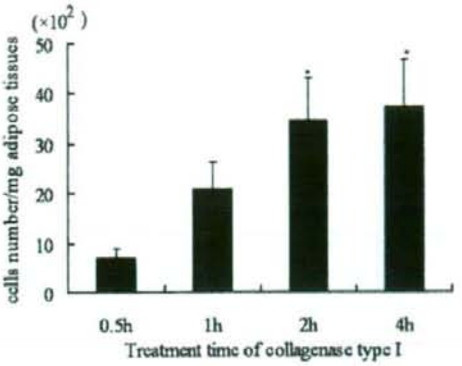

Figure 1. Time course of collagenase type I treatment for the preparation of ATSCs obtained from adipose tissue. The yields of ATSCs were increased by the treatment of the adipose tissue with the collagenase type I during the first 2 h. Student's *t*-test, $^*p < 0.005$ vs. 0.5 h and 1 h ($n = 4$).

Selection of Optimal Media Variation From Serum Concentrations for the Expansion of ATSCs

To define the optimal medium for the expansion of ATSCs with various concentrations of serum, passage two cells were plated at 5.0×10^3 cells in 48-well plates and incubated in MEM, DMEM, RPMI1640, or DMEM/F12. When ATSCs were cultured with MS, DMEM/F12 proved to be an optimal medium (Figure 2, upper panels). In contrast, when the ATSCs were cultured with FBS, DMEM was found to be the optimal medium (Figure 2, lower panels). However, the cells did not grow in each of the mediums when concentrations of both MS and FBS were lowered (1%).

ATSCs Growth on Growth Factor-Bound HCPS-Coated Plates

HCPS binding on the collagen type I substrata was tested for its ability to stimulate the activity of FGF-2, $VEGF_{165}$, or HGF on the proliferation of ATSCs (Figure 3). FGF-2 was found to be effective in stimulating ATSCs growth in a concentration-dependent manner. ATSCs growth was especially stimulated on HCPS-bound, collagen-coated plates when using FGF-2 at concentrations of 3 ng/mL or greater. $VEGF_{165}$ and HGF did not stimulate proliferation of ATSCs. Furthermore, we examined whether the growth of the cells was changed by the concentration of FGF-2 over a 3-day period [Figure 3(D)]. The concentrations of FGF-2 ranged from 0 to 1000 ng/mL, with 10% MS used for comparison. When 10 ng/mL of FGF-2 was added, growth of the cells was identical to that seen when using 10% MS. Figure 4(A) shows microphotographs of the ATSC growth on FGF-2 immobilized HCPS-bound collagen type I with DMEM/F12 medium containing 1% MS. As compared with the conditions that used both 10% MS and FBS, the cultured ATSCs had similar fibroblast-like morphology on FGF-2 immobilized HCPS-bound collagen type I with DMEM/F12 medium containing 1% MS (Figure 4).

Differentiation Assays

To determine ATSCs' potential ability for adipogenic, osteogenic, and chondrogenic phenotype cell differentiations, ATSCs were cultured under a specific condition for

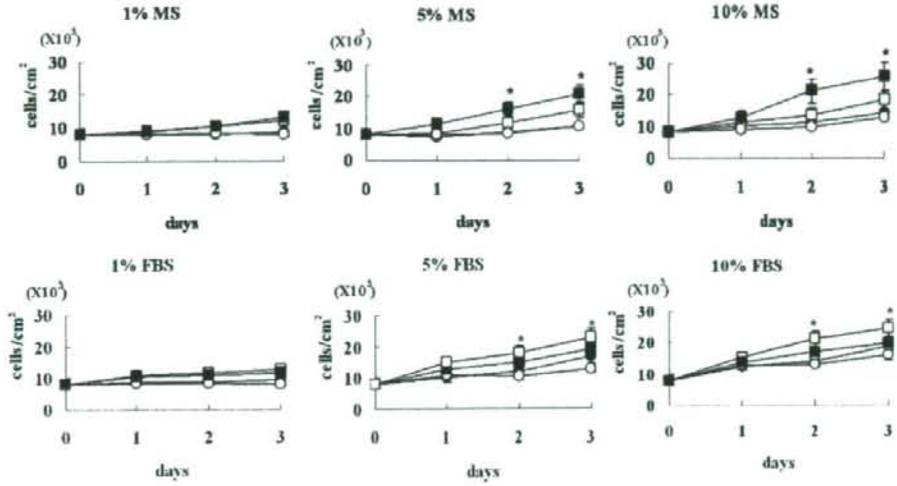

Figure 2. Optimal medium for the culture of ATSCs. The ATSCs were cultured with MEM (●), RPMI1640 (○), DMEM (□), and DMEM/F12 (■) media using the indicated concentrations of serums. Each medium was compared using Student's *t*-test. $n = 6$, $^*p < 0.05$.

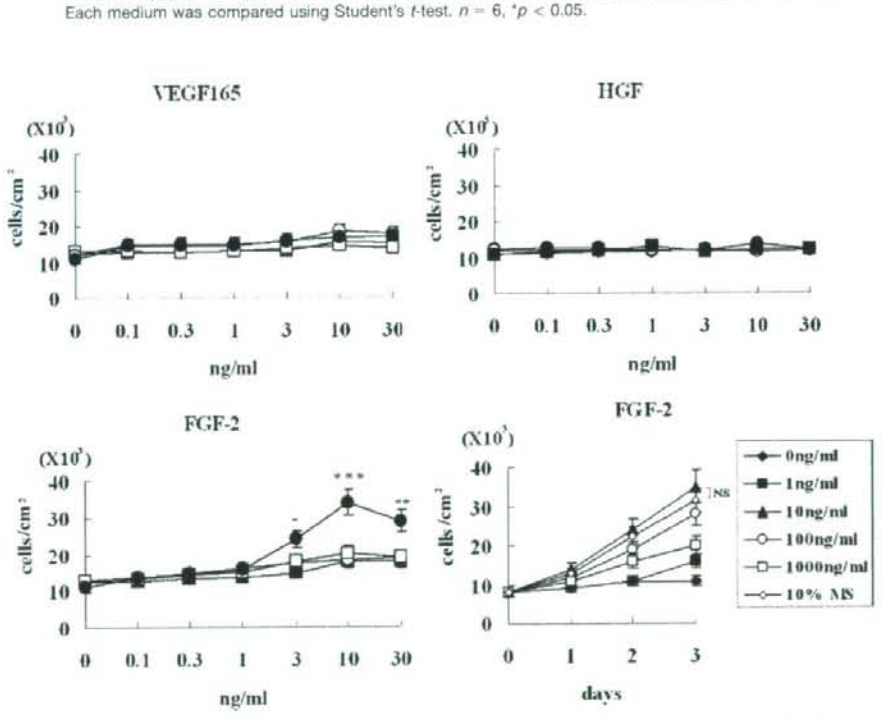

Figure 3. Effect of either immobilized FGF-2, VEGF$_{165}$, or HGF on collagen type I substrata for the enhancement of ATSCs growth. ATSCs were plated onto HCPS-bound, collagen type I-coated plates (●), HCPS-coated plates (○), collagen type I-coated plates (□), and noncoated plates (■). A–C: The cells were cultured with the indicated concentration of FGF-2, VEGF$_{165}$, or HGF in the DMEM/F12 containing 1% MS for 3 days. D: The growth of ATSCs on HCPS-bound, collagen type I-coated plates with various concentrations of FGF-2. Student's *t*-test, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, NS, not significant, ($n = 6$).

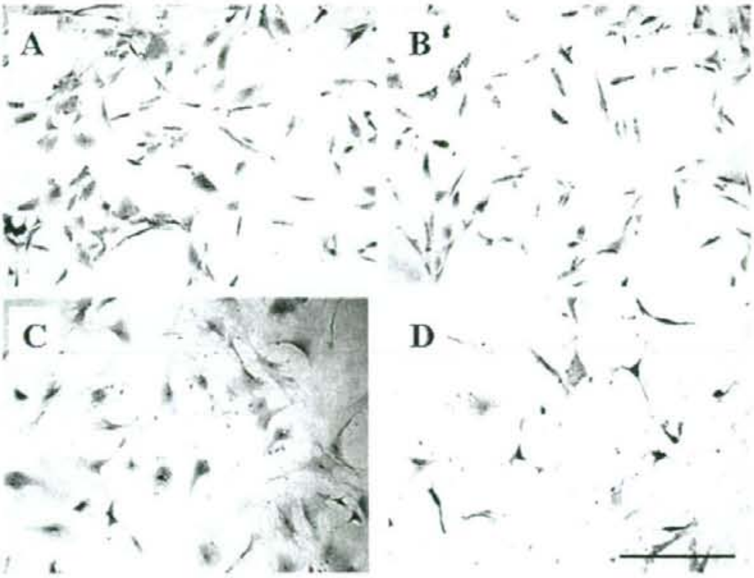

Figure 4. Morphological appearances of ATSCs (hematoxylin staining). A: ATSCs were plated with DMEM/F12 medium containing 1% MS onto FGF-2 immobilized HCPS-bound collagen type I substratum. B: ATSCs were plated with DMEM/F12 medium containing 10% MS. C: ATSCs were plated with DMEM containing 10% FBS. D: ATSCs were plated with DMEM/F12 medium (no serum). The bar represents 10 μm. The photographs were representatives of six independent samples. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

each type of differentiation. For the adipogenic assay, after cyclic induction with adipogenic medium, ATSCs had cytoplasmic lipid droplet accumulation and became adipogenic phenotype cells.[13] Historical examination revealed the formation of small lipid droplets in some cultures after two cycles of treatment. During the culturing, the number of

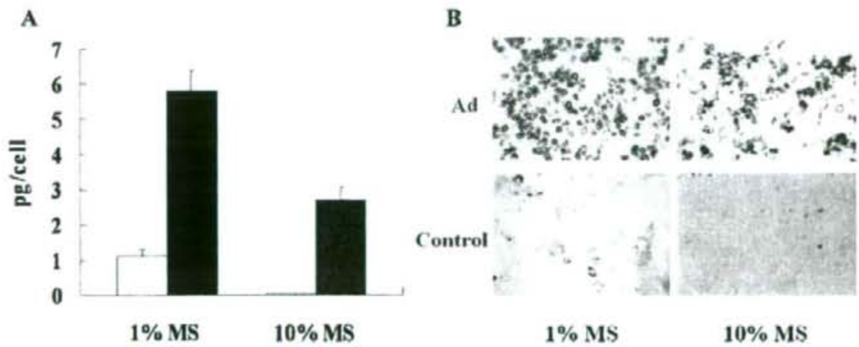

Figure 5. Adipogenic differentiation of ATSCs *in vitro*. ATSCs were expanded in DMEM/F12 medium containing 1% MS on the FGF-2 immobilized HCPS-bound collagen type I substratum (1% MS). ATSCs were expanded in DMEM/F12 medium containing 10% MS (10% MS). A: Adiponectin secretion into culture media. The cells were cultured for 10 days in the control medium (open columns) and in the adipogenic differentiation medium (closed columns). Protein levels of adiponectin were monitored by ELISA. B: Oil red O staining of ATSCs after adipogenic induction in the adipogenic medium (Ad) and the control medium (Control) after 10 days. The photographs were representatives of six independent samples. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

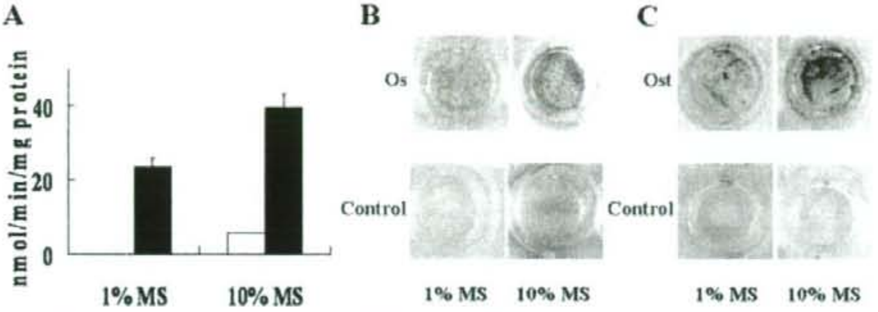

Figure 6. Osteogenic differentiation of ATSCs *in vitro*. ATSCs were expanded in DMEM/F12 medium containing 1% MS on the FGF-2 immobilized HCPS-bound collagen type I substratum (1% MS). ATSCs were expanded in DMEM/F12 medium containing 10% MS (10% MS) A: The cells were cultured for 7 days with control medium (open columns) and osteogenic medium (closed columns). ALP activity was measured as described in the Material and Methods. B: ATSCs were stained for ALP activity after osteogenic induction in osteogenic medium (Os) and control medium (Control) after 7 days. C: Alizarin red S staining for mineralization. The cells were then cultured for 14 days with osteogenic medium containing 50 μM ascorbate-2-phosphate, 10 mM β-glycerophosphate (Ost), and 10% MS, and the stained with alizarin red S. The photographs were representatives of six independent samples. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

lipid-droplet-containing cells increased. The lipid-droplet-containing cells were stained with oil red O [Figure 5(B)]. Ten days later, adiponectin secretion into the medium was examined with ELISA. The adiponectin secretion into the medium was increased by the adipogenic medium [Figure 5(A)]. For the osteogenic assay, ATSCs were cultured in osteogenic medium with BMP-2. ALP activity and ALP staining was observed [Figure 6(A,B)]. When ATSCs were cultured for 14 days in the presence of BMP-2, ascorbic acid, and β-glycerophosphate, they formed abundant mineralization in the culture [Figure 6(C)]. For the chondrogenic assay, ATSCs were cultured in the aggregate culture, using a defined medium that contained TGF-β.[14] During the culturing, the size of the pellet increased to diameters that were larger than 1 mm [Figure 7(A)]. After 21 days of culture, the pellet sections demonstrated the formation of cartilage-like tissue, as is shown by the toluidine blue staining [Figure 7(B)]. These results suggest that when using the low serum (1%) culturing method, ATSCs possess the capacity for adipogenic, osteogenic, and chondrogenic phenotype cell differentiations.

DISCUSSION

Numerous studies have demonstrated that culture-expanded ATSCs can be directed to differentiate into various types of cells, including adipocytes,[15] osteoblasts,[15–17] and chon-

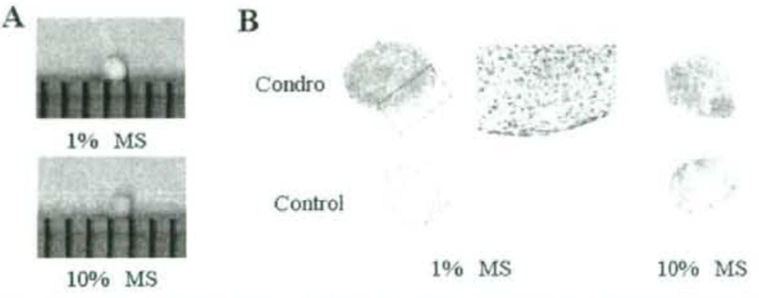

Figure 7. Chondrogenic differentiation of ATSCs *in vitro*. ATSCs were expanded in DMEM/F12 medium containing 1% MS on the FGF-2 immobilized HCPS-bound collagen type I substratum (1% MS). ATSCs were expanded in DMEM/F12 medium containing 10% MS (10% MS). A: The cells were then pelleted and cultured in chondrogenic medium for 21 days. A 1-mm scaled ruler is shown. B: Toluidine blue staining in pellet cultures *in vitro* in chondrogenic medium (chondro) and control medium (control). The photographs were representatives of four independent samples. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

drocytes,[18] both *in vivo* and *in vitro*. However, the standard growth medium for ATSCs contains about 10% FBS, and FBS is strongly immunogenic in both rodents and humans.[19] For mass culture methods used for cell-based therapies, it may be necessary to use autologous serum concentrations that are as low as possible. In this study, we evaluated allogenic (mouse) serum instead of autogenous serum to show the possibility of the usages of allogenic (mouse) serum in this study using mouse, since it was difficult to prepare enough amount of autogenous serum. We were able to effectively expand ATSCs using the allogeneic serum with concentrations as low as 1% on FGF-2 immobilized HCPS-bound collagen type I with a DMEM/F12 medium. In addition, the expanded ATSCs maintained their multipotential differentiation ability, that is, they were able to differentiate into adipocytes, osteoblasts, and chondrocytes.

Peister et al. reported that the optimal culture medium was different in MSCs obtained from different mice strains.[20] Therefore, we were examined whether the optimal medium of ATSCs was changed depending upon serum differences. The optimal medium for the ATSCs culture when using MS was DMEM/F12, and for FBS was DMEM (Figure 2). However, effective cell growth could not achieved when DMEM/F12 with 1% MS was used for the ATSCs culture.

Heparin is well known to bind various growth factors and cytokine, which both protects them and stimulates their biological activity. We previously reported that HCPS was adsorbed on collagen type I surfaces and exhibited an ability to immobilize growth factors such as FGF-2 and $VEGF_{165}$. Thus, the growth of fibroblasts or endothelial cells could be significantly stimulated on growth factor-immobilized HCPS-bound collagen type I.[11] The current results showed that the growth of cultured ATSCs on HCPS-bound collagen type I substratum in combination with FGF-2 with 1% MS was significantly stimulated, and that the growth rate was identical to when 10% MS was used. However, the addition of FGF-2 cannot induce the ATSCs proliferation in DMEM/F12 supplemented with 1% MS without HCPS-coating (data not shown). Thus, the HCPS-coating is important for maintaining the cell viability with FGF-2. The optimal concentration for FGF-2 was 10 ng/mL. In addition, ATSCs from C57BL/6J Jms Slc and C57BLKS/J lar-$Lepr^{db}$/$Lepr^{db}$ (Japan SLC, Inc.) were similarly multiplied by this method (data not shown).

FGF-2 is known to stimulate the differentiation into adipocytes and chondrocytes from mesenchymal stem cells.[21,22] The proliferation of osteoblasts and their progenitor cells is stimulated by FGF-2.[23] It is possible that the expanded ATSCs in the presence of FGF-2 may lose the multi-differentiation potential ability. Therefore, we carried out the differentiation study to show the abilities of differentiations in the expanded ATSCs using each differentiation medium. Our results clearly showed that expanded ATSCs on HCPS-bound collagen type I substratum in combination with FGF-2 with 1% MS did keep their abilities to differentiate into adipocytes, osteoblasts, and chondrocytes *in vitro*.

In conclusion, we established a method for expanding ATSCs under low serum conditions (1% MS concentration), using FGF-2 immobilized HCPS-bound collagen type I substratum. The expanded ATSCs maintained their potential to differentiate into adipocytes, osteoblasts, and chondrocytes. These results suggest that the expanded ATSCs on FGF-2 immobilized HCPS-bound collagen type I substratum with DMEM/F12 medium containing less than 1% allogeneic serum may provide a promising cell source, especially in the preparation of large amounts of ATSCs that are needed for cell-based therapies in several clinical fields.

REFERENCES

1. Zuk PA, Zhu M, Ashjian P, De-Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Franser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 2002;13:4279–4295.
2. Schaffler A, Buchler C. Concise review: Adipose tissue-derived stromal cells—Basic and clinical implications for novel cell-based therapies. Stem Cells 2007;25:818–827.
3. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997;276:71–74.
4. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–147.
5. Spees JL, Gregory CA, Singh H, Tucker HA, Peister A, Lynch PJ, Hsu SC, Smith J, Prockop DJ. Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. Mol Ther 2004;9:747–756.
6. Martin MJ, Muotri A, Gage F, Varki A. Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat Med 2005;11:228–232.
7. Tanaka Y, Kimata K, Adams DH, Eto S. Modulation of cytokine function by heparan sulfate proteoglycans: Sophisticated models for the regulation of cellular responses to cytokines. Proc Assoc Am Phys 1998;110:118–125.
8. Jackson RL, Busch SJ, Cardin AD. Glycosaminoglycans: Molecular properties, protein interactions, and role in physiological processes. Physiol Rev 1991;71:481–539.
9. Ishihara M, Ono K, Ishikawa K, Hattori H, Saito Y, Yura H, Akaike T, Ozeki Y, Tanaka S, Mochizuki H, Kurita A. Enhanced ability of heparin-carrying polystyrene (HCPS) to bind to heparin-binding growth factors and to inhibit growth factor-induced endothelial cell growth. J Biochem 2000;127:797–803.
10. Ishihara M, Sato M, Hattori H, Saito Y, Yura H, Ono K, Masuoka K, Kikuchi M, Fujikawa K, Kurita A. Heparin-carrying polystyrene (HCPS)-bound collagen substratum to immobilize heparin-binding growth factors and to enhance cellular growth. J Biomed Mater Res 2001;56:536–544.
11. Ishihara M, Saito Y, Yura H, Ono K, Ishikawa K, Hattori H, Akaike T, Kurita A. Heparin-carrying polystyrene to mediate cellular attachment and growth via interaction with growth factors. J Biomed Mater Res 2000;50:144–152.
12. Kadowaki A, Tsukazaki T, Hirata K, Shibata Y, Okubo Y, Bessho K, Komori T, Yoshida N, Yamaguchi A. Isolation and characterization of a mesenchymal cell line that differentiates

into osteoblasts in response to BMP-2 from calvariae of GFP transgenic mice. Bone 2004;34:993–1003.
13. Hausman DB, DiGirolamo M, Bartness TJ, Hausman GJ, Martin RJ. The biology of white adipocyte proliferation. Obes Rev 2001;2:239–254.
14. Barry F, Boynton RE, Liu B, Murphy JM. Chondrogenic differentiation of mesenchymal stem cells from bone marrow: Differentiation-dependent gene expression of matrix components. Exp Cell Res 2001;268:189–200.
15. Hattori H, Ishihara M, Fukuda T, Suda T, Katagiri T. Establishment of a novel method for enriching osteoblast progenitors from adipose tissues using a difference in cell adhesive properties. Biochem Biophys Res Commun 2006;343:1118–1123.
16. Hattori H, Sato M, Masuoka K, Ishihara M, Kikuchi T, Matsui T, Takase B, Ishizuka T, Kikuchi M, Fujikawa K, Ishihara M. Osteogenic potential of human adipose tissue-derived stromal cells as an alternative stem cell source. Cells Tissues Organs 2004;178:2–12.
17. Hattori H, Masuoka K, Sato M, Ishihara M, Asazuma T, Takase B, Kikuchi M, Nemoto K, Ishihara M. Bone formation using human adipose tissue-derived stromal cells and a biodegradable scaffold. J Biomed Mater Res B 2006;76:230–239.
18. Masuoka K, Asazuma T, Hattori H, Yoshihara Y, Sato M, Matsumura K, Matsui T, Takase B, Nemoto K, Ishihara M. Tissue engineering of articular cartilage with autologous cultured adipose tissue-derived stromal cells using atelocollagen honeycomb-shaped scaffold with a membrane sealing in rabbits. J Biomed Mater Res B 2006;79:25–34.
19. Selvaggi TA, Walker RE, Fleisher TA. Development of antibodies to fetal calf serum with arthus-like reactions in human immunodeficiency virus-infected patients given syngeneic lymphocyte infusions. Blood 1997;89:776–779.
20. Peister A, Mellad JA, Larson BL, Hall BM, Gibson LF, Prockop DJ. Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. Blood 2004;103:1662–1668.
21. Neubauer M, Fischbach C, Bauer-Kreisel P, Lieb E, Hacker M, Tessmar J, Schulz MB, Goepferich A, Blunk T. Basic fibroblast growth factor enhances PPARγ ligand-induced adipogenesis of mesenchymal stem cells. FEBS Lett 2004;577:277–283.
22. Olney RC, Wang J, Sylvester JE, Mougey EB. Growth factor regulation of human growth plate chondrocyte proliferation in vitro. Biochem Biophys Res Commun 2004;317:1171–1182.
23. Globus RK, Patterson-Buckendahl P, Gospodarowicz D. Regulation of bovine bone cell proliferation by fibroblast growth factor and transforming growth factor beta. Endocrinology 1988;123:98–105.

Liposome-Encapsulated Hemoglobin Transfusion Rescues Rats Undergoing Progressive Hemodilution From Lethal Organ Hypoxia Without Scavenging Nitric Oxide

Yashiro Nogami, MD, Manabu Kinoshita, MD,† Bonpei Takase, MD,‡ Yoshitaka Ogata, PhD,§ Daizoh Saitoh, MD,† Makoto Kikuchi, PhD,¶ Masayuki Ishihara, PhD,‡ and Tadaaki Maehara, MD**

Objective: To investigate the efficacy of liposome-encapsulated hemoglobin (LHb) transfusion in rats undergoing lethal progressive hemodilution.

Summary Background Data: Unlike other acellular hemoglobin-based oxygen carriers, LHb has lipid bilayer membranes that are similar to mammalian red blood cells (RBCs), which prevent hemoglobin from having any direct contact with the blood components and the endothelium. Acellular hemoglobin has a high affinity for nitric oxide (NO), and because they are reported to behave as NO scavengers, acellular hemoglobin-based oxygen carriers could have pressor effects on the peripheral vessels. During a massive hemorrhage, acellular hemoglobin caused vasoconstriction could decrease peripheral perfusion, thereby leading to diminished oxygen delivery.

Methods: Rats were subjected to blood withdrawal (0.2 mL/min) with a simultaneous resuscitation using an isovolemic fluid transfusion that contained LHb, 5% albumin, or washed rat RBCs for 150 minutes (n = 15 in each group).

Results: All rats transfused with LHb or RBCs were rescued from lethal progressive hemodilution, whereas none of the albumin-transfused rats survived. LHb did not affect the plasma NO metabolite levels, suggesting it was not a potent NO scavenger. LHb also improved hemodilution-induced metabolic acidosis, and reduced exaggerated neuroendocrine responses and injuries to the heart, liver, and kidney. It suppressed expression of hypoxia-inducible factor-1alpha in the liver and kidney, suggesting improvement of hypoxia at molecular response levels. However, neither transfused LHb nor RBCs improved the acute lung injury that occurs after progressive hemodilution.

Conclusion: LHb transfusion is effective in rescuing rats undergoing progressive hemodilution from lethal organ hypoxia without scavenging NO.

(*Ann Surg* 2008;248: 310–319)

From the Departments of *Surgery and ¶Medical Engineering, and Divisions of †Traumatology and ‡Biomedical Engineering, National Defense Medical College Research Institute, Tokorozawa, Japan; §Terumo Research Institute, Kanagawa, Japan.

Supported in part by a grant-in-aid for the Special Research Program from the National Defense Medical College to B.T.

Reprints: Manabu Kinoshita, Division of Traumatology, National Defense Medical College Research Institute, 3-2 Namiki, Tokorozawa, 359-8513, Japan. E-mail: manabu@ndmc.ac.jp.

ISSN: 0003-4932/08/24802-0310

DOI: 10.1097/SLA.0b013e3181820c80

Recently, several modified hemoglobins (Hbs) that transport oxygen have been developed as blood substitutes. Despite many studies demonstrating the efficacy of diaspirin cross-linked Hb (DCLHb) in hemorrhagic shock,[1,2] Sloan et al reported that the use of DCLHb was accompanied by an unexpected increase in mortality.[3] It has also been reported that polymerized Hb solution did not increase survival in the rat hemorrhagic shock model,[4] even though there have been several clinical trials studying polymerized Hb.[5,6] DCLHb and polymerized Hb, which are the so-called acellular Hbs, have been reported to act as nitric oxide (NO) scavengers and to have pressor effects on the peripheral vessels.[7,8] To increase its overall molecular size and attenuate vasoconstriction, the surface of the Hb was modified with polyethylene glycol (PEG).[9,10] Although clinical trials of PEGylated hemoglobin (PEG-Hb) are currently ongoing,[11] PEG-Hb is still structurally quite different from mammalian red blood cells (RBCs) because it does not have a membrane that encapsulates the Hb. Therefore, we encapsulated the Hb using a lipid bilayer membrane (liposome) that prevents direct contact with the blood components and the endothelium, resulting in the shielding of the endothelium from the side-effects of acellular Hb.[12] Liposome-encapsulated Hb (LHb), with the surface of the liposome modified by PEG, does not provoke vasoconstriction, and has an oxygen transporting capability equivalent to mammalian RBCs.[12–16] In the present study, we demonstrated that LHb transfusion is capable of rescuing rats from lethal progressive hemodilution by improving the tissue hypoxia, without scavenging NO in the circulation.

MATERIALS AND METHODS

Animals and Surgical Preparation

This study was conducted in accordance with the guidelines for the care of animal subjects, as defined by the Institutional Review Board at the National Defense Medical

College, Tokorozawa, Japan. Male Sprague-Dawley rats (200–250 g, Japan SLC Inc., Tokyo) were studied. After anesthetization using an intraperitoneal injection with pentobarbital (50 mg/kg, Abbott Labs, Chicago, IL), the rats were intubated and sedated with 1.5% sevoflurane-mixed air inhalation (Maruishi Pharm, Osaka, Japan) using a vaporizer (Shinano Mfg. Co., Ltd., Tokyo), followed by mechanical ventilation (tidal volume; 3 mL, respiratory rate; 80/min). Surgical catheters (polyethylene indwelling needle 27G, Terumo Co., Tokyo) were inserted into the femoral artery and vein of each rat. Rat body temperature was maintained at 37–38°C by a heating pad.

Preparation of LHb, Washed Rat RBCs and 5% Albumin Solution

The LHb was prepared at the Terumo Research and Development Center (Terumo Co., Tokyo).[12] Briefly, purified human Hb solution was prepared from outdated human RBCs provided by the Japanese Red Cross. After washing, human RBCs were hemolyzed with simultaneous virus inactivation. Subsequently, Hb was concentrated using a reverse osmosis membrane and sterilized. Thereafter, the purified Hb was adjusted to 40 to 50 mm Hg of P_{50} (the oxygen partial pressure at which hemoglobin is half saturated with oxygen) by adding inositol hexaphosphate. After adjusting the P_{50}, purified Hb was encapsulated with lipid ingredients through the use of high-speed emulsification. The surface of the encapsulating lipid membrane was then modified with 5-kDa polyethylene glycol. The LHb was diluted with saline to achieve a final Hb concentration of 6 g/dL and then deoxygenated with N_2 bubbling for storage. The diameters of LHb were around 220 nm. The total lipid concentration of the LHb solution was 3.9 g/dL, with the methemoglobin proportion at 6.3%. The content of lipopolysaccharide was less than 0.1 EU/mL.[12] After administration of LHb, estimations of the pharmacokinetics of LHb were made based on the altered concentration of the human Hb that was used as a marker of LHb in the rat blood. The concentration of human Hb was determined by enzyme-linked immunosorbent assay (ELISA), which specifically detects human Hb but does not react with rat Hb. As a result, the biologic half-life of LHb was approximately 13 hours in rats (S. Kaneda, PhD, Terumo Research and Development Center, Terumo Co., Kanagawa, Japan, 2007, personal communication).

To prepare washed rat RBCs, a syringe containing citrate-phosphate-dextrose-adenine solution was used to collect blood from donor Sprague-Dawley rats. The blood was then centrifuged at 3,000× g for 10 minutes at 4°C. The centrifuged RBCs were washed and diluted with saline to achieve a final concentration of 6 g/dL.[15] Before use, human serum albumin (HSA, Kaketsuken, Kumamoto, Japan) was added to both the LHb and rat RBC suspensions to achieve a final albumin concentration of 5 g/dL (colloid osmotic pressures = 20 mm Hg).[17] A 5% HSA solution (5 g/dL) was also prepared.

Massive Hemorrhage Model and Isovolemic Fluid Resuscitation

Blood was continuously withdrawn from rats via the femoral artery at 0.2 mL/min. Simultaneous fluid resuscitation of the hemorrhaging rats (n = 10) was performed by intravenous injection via the femoral vein with a 5% albumin solution at 0.2 mL/min. Control hemorrhaging rats (n = 10) did not receive any fluid resuscitation.

Lethal Progressive Hemodilution Model

Rats were subjected to progressive hemodilution by exchange transfusion for 150 minutes. In rats, blood was continuously withdrawn from the femoral artery at 0.2 mL/min with a simultaneous transfusion performed by administering an intravenous injection of LHb, rat RBCs, or a 5% albumin solution into the femoral vein at 0.2 mL/min (n = 15 in each group).

Measurements of Hemodynamics and Hematological Parameters

The mean arterial pressure (MAP) and heart rate were measured from the cannulated femoral artery with a polygraph recording system (model RM-6000, Nihon Kohden, Tokyo). Blood samples were also collected from the femoral artery. The RBC count, Hb concentration, hematocrit, and the white blood cell (WBC) count were measured using a hematology analyzer (PCE 170, Erma Inc., Tokyo).

Measurement of Hemoglobin in Rat Blood Samples Containing LHb and RBCs

Hb concentration in LHb could not be accurately determined, because the liposome capsules interfered with the spectrophotometric measurement of Hb absorbance. However, the measured Hb concentration in the LHb solution, as determined by the Erma PCE 170 hematology analyzer, was 5-fold higher than the actual concentration that was determined when using a specific ELISA for human Hb during the production of LHb. We then estimated the actual Hb concentration in the LHb-transfused rat samples based on the measured Hb concentration of each sample. First, the rat RBC-derived Hb concentration in an LHb-mixed blood sample was estimated by reproducing a RBC suspension that had the same hematocrit as the LHb-mixed blood sample. We then measured its Hb concentration, as the hematocrit in the LHb-mixed blood sample reflected the concentration of the rat RBCs since the MCV (mean corpuscular volume) essentially remained unchanged in all of the rats during the experiment. The LHb-derived Hb concentration was then obtained by subtracting the rat RBC-derived Hb concentration from the measured Hb concentration, with the determination of the actual LHb-derived Hb concentration calculated by dividing the obtained LHb-derived Hb concentration by 5. The total concentration of Hb in the samples containing a mixture of RBCs and LHb was determined by adding the estimated LHb-derived Hb concentration and the rat RBC-derived Hb concentration.

Measurements of Blood Gas Parameters

The arterial blood oxygen and carbon dioxide tensions, pH, and base excess were determined using a pH/blood gas analyzer (IL Synthesis 25 Critical Care Analyzer, Instrumentation Laboratory Co., Lexington, MA). The alveolar-arterial oxygen tension difference ($AaDO_2$) was calculated using the following formula:

$AaDO_2$ (mm Hg) = $[FiO_2\ ((P_{BAR} - P_{H_2O})]\text{-}PaCO_2/R\text{-}PaO_2$, where FiO_2 (inspired oxygen concentration) = 0.21; P_{BAR} (barometric pressure) = 760 mm Hg; P_{H_2O} (water vapor pressure) = 47 mm Hg; R (respiratory quotient) was assumed to be 0.83; and the $PaCO_2$ (arterial partial pressure of carbon dioxide) and the PaO_2 (arterial partial pressure of oxygen) were measured using the blood gas analyzer.

Measurements of Plasma NOx Concentrations

The collected blood samples were immediately heparinized and then centrifuged at 50,000× *g* at 4°C for 20 minutes to remove the LHb particles.[18] The plasma supernatant was stored at −80°C until the analytic procedure. After thawing, the plasma was mixed with methanol (1:1) followed by centrifugation at 10,000× *g* at 4°C for 10 minutes to remove the proteins. Determination of plasma NOx was performed by a high-performance liquid chromatography-Griess system (ENO-20, Eicom, Kyoto, Japan).[19,20] This system used the postcolumn derivatization method, which employed Griess reagents for the detection of nitrite and nitrate. Nitrite and nitrate were first separated from the other substances on the separation columns. Thereafter, nitrite reacted with the Griess reagent and generated diazo compounds. Nitrate was reduced by a cadmium-copper column for the reaction with the Griess reagent. A visible detector installed in the column oven was used to measure the amount of diazo compound that was present. The limits of detection and sensitivities were 0.1 μM for both the NO_2^- and NO_3^-. The plasma loading volume was 10 μL. To calculate the NO_2^- and NO_3^- quantification, PowerChrom analytical software (AD Instrument Co., Ltd., Tokyo) was used to determine the areas under the curves (AUCs) for each of the chromatograms.[21] NOx was the sum of the NO_2^- and NO_3^- concentrations.

Measurements of Plasma and Serum Parameters

Collected blood samples were centrifuged at 50,000× *g* for 20 minutes.[18] The resulting plasma or sera were stored at −80°C until assayed. Plasma lactate concentrations were measured by clinical chemistry testing (SRL, Tokyo). Plasma epinephrine and norepinephrine concentrations were measured by the 3-Cat Enzyme Immunoassay (Labor Diagnostika Nord GmbH & Co. KG, Nordhorn, Germany). Plasma C3a concentrations were measured by a Rat C3a ELISA kit (Cedarlane Laboratory Ltd., Ontario, Canada). Serum creatinine and alanine aminotransferase (ALT) concentrations were measured by a FUJI dry-chem systems (Fuji Film, Tokyo). Serum heart-type fatty acid-binding protein concentrations were measured by a commercially available ELISA kit specific for rat/mouse H-FABP (HyCult Biotechnology b.v., Uden, Netherlands).

Measurements of Left Ventricular Ejection Fractions

Two-dimensionally guided M-mode recordings were obtained from the short-axis view at the level of the papillary muscles using an FF sonic UF-750XT ultrasound system and a FUT-LD386-9A 9.0-M Hz linear-array transducer (Fukuda Denshi, Tokyo). Left ventricular (LV) end-systolic and end-diastolic dimensions, as well as systolic and diastolic wall thickness, were measured from the M-mode tracings using the leading-edge convention of the American Society of Echocardiography. For each M-mode measurement, at least 3 consecutive cardiac cycles were sampled. LV mass and ejection fraction were calculated from the short axis wall thickness and chamber dimension measurements by assuming a spherical LV geometry.[22]

Measurements of Protein Concentration in Bronchoalveolar Lavage Fluid (BALF) and the Lung Wet/Dry Ratio

The rats were euthanized for removal of the lungs en bloc. Immediately after removal, 2 mL saline was slowly infused through the primary bronchus to inflate the lung, and then 1.2 mL of the lavage fluid was gently withdrawn and stored at −80°C until assayed.[23] The protein concentration in BALF was measured by the DC (Detergent Compatible) Protein Assay (Bio-Rad Laboratory. Inc., Hercules, CA). The wet/dry weight ratio of the lung was estimated to determine the water content of the lung. Rat lungs were dissected free of nonpulmonary tissue, weighed, and then dried in an oven until a constant weight was achieved. The wet/dry ratio was obtained by dividing the wet weight by the final dried weight.[24]

Histologic Examinations

The rats were euthanized to remove the spleens, livers, kidneys, and lungs at the end of the exchange transfusion period. The lungs were immersed in 20% formalin for 2 days, after gentle intratracheal instillation of 20% formalin with a pressure of about 10 cm H_2O. Other organs were also immersed in 20% formalin for 2 days. Slides were prepared from the fixed specimens and stained with hematoxylin and eosin.

Immunohistochemistry of Hypoxia-Inducible Factor-1alpha (HIF-1alpha) Protein

After deparaffinization, specimen slides were placed in 3% H_2O_2 in phosphate buffered saline (PBS) for 10 minutes. To unmask antigen, the slides were incubated in 50 mmol/L Tris buffer containing 0.2 mmol/L EDTA (pH 9.0) at 60°C for 16 hours in a covered water bath. After incubation with 1.5% normal goat serum in PBS for 30 minutes, the slides were incubated with purified mouse anti-HIF-1alpha antibody (Novus Biologicals, Littleton, CO) diluted 1:2,000 in 1.5% normal goat serum in PBS for 90 minutes at room temperature. The slides were washed and then incubated with peroxidase conjugated donkey antimouse IgG (AP-192P, Chemicon International Inc., Temecula, CA) diluted 1:100 in PBS for 30 minutes at room temperature. Reactions were visualized with 3, 3'-diaminobenzidine (DAB), and counterstained with hematoxylin. For the negative control, the incubation step with the primary antibody was omitted.

Statistical Analysis

Statistical analyses were performed using the Stat View 4.02J software package (Abacus Concepts, Berkeley, CA). Survival rates were compared by the Wilcoxon signed rank test. Statistical evaluations between 2 groups were compared using the Student *t* test, and any other statistical evaluations were compared using the one-way analysis of variance,

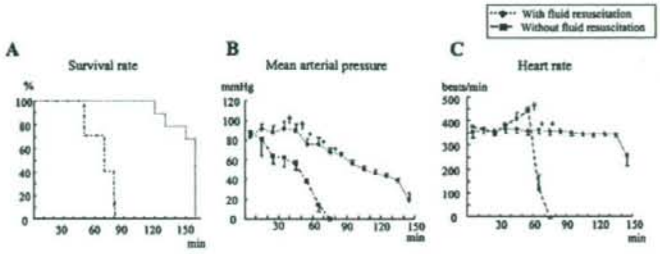

FIGURE 1. Comparisons between rats with and without fluid resuscitation during a period of continuous blood withdrawal. The effect of normotensive fluid resuscitation on survival (A), mean arterial pressure (B) and heart rates (C) in a progressive isovolemic exchange model in rats are shown. Rats underwent blood withdrawal at 0.2 mL/min via the femoral artery, simulating an uncontrolled hemorrhage. The rats simultaneously underwent normotensive fluid resuscitation with 5% albumin at 0.2 mL/min via the femoral vein. Untreated hemorrhaging rats did not receive any fluid resuscitation. The data are the mean ± SE from 10 rats (A, B, and C) in each group. *$P < 0.01$, †$P < 0.05$ versus the other group.

followed by the Bonferroni post hoc test. Data are presented as the mean ± SE, with $P < 0.05$ considered statistically significant.

RESULTS

Normotensive Fluid Resuscitation Does Not Rescue Rats From Massive Hemorrhage Due to Fatal Hypohemoglobinemia

Hemorrhagic shock was induced in rats by continuous blood withdrawal (0.2 mL/min). In all rats that did not receive any type of resuscitation, death occurred within 80 minutes due to hypovolemic shock (Fig. 1A). These animals developed decreased MAP and increased heart rates (Figs. 1B, C). In the rats simultaneously undergoing isovolemic fluid resuscitation with 5% albumin (0.2 mL/min), all animals survived beyond 80 minutes (Fig. 1A). However, due to severe fatal anemia (Hb concentration: <2 g/dL), none of the rats in the 5% albumin group survived beyond 160 minutes.

LHb Transfusion Can Rescue Rats From Progressive Hemodilution Without Decreasing Plasma NOx Concentrations

Although all of the LHb-transfused rats and the RBC-transfused rats survived more than 48 hours after hemodilution, none of the 5% albumin-transfused rats survived (Fig. 2A). For both the LHb- and RBC-transfused rats, the MAP was maintained during the period of exchange transfusion (Fig. 2B). For all 3 groups, the heart rates were maintained up until just before the end of the exchange transfusion period (Fig. 2C), which suggests that there was successful isovolemic fluid resuscitation. Although the decreases seen in the RBC counts and hematocrits were similar for both the LHb- and albumin-transfused rats (Figs. 2D, E), the LHb-transfused rats and RBC-transfused rats exhibited higher Hb concentrations than those that were observed for the albumin-transfused rats beyond 90 minutes from the start of the exchange transfusion (Fig. 2F) (Hb concentrations in LHb-transfused rats were estimated as previously described). When compared with the albumin-transfused rats, the LHb-transfused rats had significantly higher WBC counts at the end of the exchange transfusion period. There were no differences in the WBC counts observed between the LHb-transfused rats and the RBC-transfused rats (Fig. 2G). The effect of LHb transfusion on plasma NOx levels was examined before the hemodilution, at 60, and 150 minutes. The plasma NOx concentrations were similar for the LHb-, RBC- and albumin-transfused rats at all time points sampled (Fig. 2H).

LHb Transfusion Prevents Metabolic Acidosis, Exaggerated Neuroendocrine Response, and Multiorgan Dysfunctions Except Pulmonary Dysfunction in Rats After Progressive Hemodilution

Both LHb- and RBC-transfused rats had significantly lower plasma lactate levels than the albumin-transfused rats at the end of the exchange transfusion period. Their lactate levels were similar to the normal levels (Table 1). LHb- and RBC-transfused rats also had a similar pH and base excess, and for both groups, the pH and base excess levels were significantly higher than those of the albumin-transfused rats, even though their base excess levels were still lower than the normal levels (Table 1). As compared with the albumin-transfused rats, the LHb-transfused rats showed significantly lower plasma epinephrine and norepinephrine levels at the end of the exchange transfusion period, even though the catecholamine levels were significantly higher than those seen for the RBC-transfused rats (Table 1). Because liposomes, which are components of the LHb, have been reported to activate the complement system, we also examined the plasma C3a levels.[25] However, the LHb-transfused rats did not cause an increase in the plasma C3a levels as compared with that seen for the other transfusion groups (Table 1). Although both LHb- and RBC-transfused rats showed significantly lower serum H-FABP concentrations, and significantly higher LV ejection fractions as compared with the albumin-transfused rats, their H-FABP levels were higher than the normal levels (Table 1). H-FABP is a sensitive marker for the early detection of myocardial injury, and higher LV ejection fractions indicate the preservation of myocardial function. Similarly, both LHb- and RBC-trans-