Original Article

Tissue Regeneration through Self-Assembled Peptide Amphiphile Nanofibers

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Abstract

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Introduction: In the present study, we hypothesized that a novel approach to promote vascularization would be to create injectable three dimensional (3-D) scaffolds within growth factor that enhance the sustained release of growth factor and induce the angiogenesis.

Material and Methods: We demonstrate that a 3-D scaffold can be formed by mixing of peptide-amphiphile (PA) aqueous solution with hepatocyte growth factor (HGF) solution. PA was synthesized by standard solid phase chemistry that ends with the alkylation of the NH_2 terminus of the peptide. The sequence of arginine-glycine-aspartic acid (RGD) was included in peptide design as well. A 3-D network of nanofibers was formed by mixing HGF suspensions with dilute aqueous solution of PA.

Results: Scanning electron microscopy (SEM) examination revealed the formation of fibrous assemblies with an extremely high aspect ratio and high surface areas with mean diameter of less than 200 nm. *In vitro* HGF release profile of 3-D nanofibers was investigated while angiogenesis induced by the released HGF was being assessed. *In vivo* potential ability of PA nanofibers to induce angiogenesis was assessed through subcutaneous injection of PA solution, HGF solution, and PA in combination with HGF solutions. Injection of PA with HGF induced significant angiogenesis around the injected site, in marked contrast to HGF injection alone and PA injection alone.

Conclusion: The combination of HGF-induced angiogenesis is a promising procedure to improve tissue regeneration.

Keywords: Tissue regeneration, Controlled release, Growth factor

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Introduction

Tissue engineering is designed to regenerate natural tissues or to create biological substitutes for defective or lost organs using the cells. Considering the usage of cells in the body, it is no doubt that a sufficient supply of nutrients and oxygen to the transplanted cells is vital for their survival and functional maintenance (1). Without a sufficient supply, only a small number of cells pre-seeded in the scaffold or migrated into the scaffold from the surrounding tissue would survive. Rapid formation of a vascular network at the transplanted site of cells would be a promising way to provide cells with the vital supply.

This process of generating new microvasculature, termed neovascularization, is a process observed physiologically in development and wound healing (2). It has been recently

demonstrated that HGF has diverse potentials for proliferation, differentiation, mitogenesis, and morphogenesis of various cells (3, 4, 5). The growth factors stimulate appropriate cells (e.g., endothelial cells), already present in the body, to migrate from the surrounding tissue, proliferate, and finally differentiate into blood vessels (2). However, one cannot always expect the sustained angiogenesis activity when these proteins are only injected in the solution form, probably because of their rapid diffusional excretion from the injected site. One possible way for enhancing the *in vivo* efficacy is to achieve its controlled release over an extended time period by incorporating the growth factor in a polymer carrier. If this carrier is biodegraded, harmonized with tissue growth, it will work as a scaffold for tissue regeneration in addition to a carrier matrix for the growth factor release.

Material design of scaffold for cell proliferation and differentiation is one of the key technologies for tissue engineering. The scaffold should mimic the structure and biological function of native extracellular matrix (ECM) as much as possible, both in terms of chemical composition and physical structure. Native ECM does far more than just provide a physical support for cells. It also provides a substrate with specific ligands for cell adhesion and migration, and regulates cellular proliferation and function by providing various growth factors. It is reasonable to expect that an ECM-mimicking tissue-engineered scaffold will play a similar role to promote tissue regeneration *in vitro* as native ECM does *in vivo*. A well-known feature of native ECM structures is the nanoscaled dimensions of their physical structure. In a typical connective tissue, structural protein fibers such as collagen and elastin fibers have diameters ranging from several ten to several hundred nanometers (6). The nanoscaled protein fibers entangle with each other to form a nonwoven mesh that provides tensile strength and elasticity, and laminin, which provides specific binding site for cell adhesion, also exists as nanoscaled fibers in ECM. With the ability to form nano-fibrous structures, a drive has begun to mimic the ECM and form scaffolds that are artificial extracellular matrix suitable for tissue formation.

These nano-fibrous scaffolds attempt to mimic collagen, a natural extracellular matrix component, and could potentially provide a better environment for tissue formation in tissue engineering systems. Collagen accounts for one-third of total body proteins and is one of the main components of the ECM. Three different approaches have emerged for the formation of nano-fibrous materials: self-assembly, electrospinning, and phase separation (7).

Each approach is very different and has a unique set of characteristics as a scaffolding system. For instance, self-assembly approach can only generate small diameter nano-fibers in the lower end of the range of natural extracellular matrix collagen, while electrospinning has only generated large diameter nano-fibers on the upper end of the range of natural extracellular matrix collagen. Phase separation, on the other hand, has generated nano-fibers in the same range as natural extracellular matrix collagen and allows for the design of macropore structures. These attempts at an artificial extracellular matrix have the potential to accommodate cells and guide their growth and subsequent tissue regeneration. Self-assembly that is the autonomous organization of molecules into patterns or substrates without human intervention, is common throughout nature and technology. Self-assembly of natural or synthetic macromolecules produces nanoscaled supramolecular structures and nanofibers. Specifically designed amphiphilic peptides that contain a carbon alkyl tail and several other functional peptide regions have been synthesized and self-assembled into nanofibers with a diameter of 7.6 ± 1 nm (8). These self-assembled nanofibers have been recently used to study selective differentiation of neural progenitor cells (9). Nanoscaled fibers produced by self-assembly of amphiphilic peptides seem to have great potential application in the field of biomaterials and tissue engineering. However, to our knowledge, there is no trial to take an advantage of these self-assembled nanofibers as an injectable carrier for growth factors.

The objective of the present study is to fabricate the 3-D networks of nanofibers by mixing HGF solution with aqueous solution of amphiphilic peptides and use it for feasibility of prevascularization in improving efficiency of tissue regeneration by the HGF

release from the 3-D networks of nanofibers formed.

Material and Methods

Amino acid derivatives, derivatized resins, were purchased from Sowa Trading Co., Inc., Tokyo, Japan. Human recombinant HGF (Lot # GJ09909B) was obtained from PeproTech EC, Ltd., London, UK. Other chemicals were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan and used as soon as obtained. All water used was deionized with a Millipore Milli-Q water purifier operating at a resistance of 18 MΩ.

Synthesis of the PA

The PA was prepared on a 0.5-mmol scale by using standard fluorenylmethoxycarbonyl chemistry (F-moc) (10) on a fully automated peptide synthesizer (Peptide Synthesizer Model 90, Advanced ChemTech, Inc., KY, USA). The chemical structure of PA contains RGD (arginine-glycine-aspartic acid), a Glu residue, four Ala and three Gly residues (A_4G_3), followed by an alkyl tail of 16 carbons. PA was characterized by matrix-assisted laser desorption ionization-time of flight MS and was found to have the expected molecular weight. The similar preparation procedure was performed to prepare PA without RGD incorporation by starting fluorenylmethoxycarbonyl-Glu (OBut)-Wang resin to couple to amino acid residues.

Formation of 3-D network of self-assembled PA nanofibers

A transparent gel-like solid was formed by mixing of HGF solution with 1 wt % PA aqueous solution in a 1:1 volume ratio. Formation of nanofibers strongly depends on the particular chemical structure of PA. PA can self-assemble into sheets, spheres, rods, disks, or channels depending on the shape, charge, and environment. Amphiphiles with a conical shape in which the hydrophilic head group is somewhat bulkier than its narrow hydrophobic tail have been shown to form cylindrical micelles (8). The PA synthesized contains a hydrophobic domain of 16 carbons and a hydrophilic domain of A_4G_3, a Glu residue, following a conical shape made of RGD. The Glu residue provides a negative net charge at pH 7.4 for PA, and the positively-charged HGF can screen electrostatic repulsion among them. Thus once electrostatic repulsions are reduced, the molecules are driven to assemble by hydrogen bond formation and unfavorable contact among hydrophobic segments and water molecules.

Morphological observation

The morphology of self-assembled PA nanofibers was observed using a scanning electron microscope (SEM, S-2380N; Hitachi, Tokyo, Japan). The sample was obtained by network dehydration and critical point drying of samples caged in a metal grid to prevent network collapse. The dried sample was coated with gold on an ion sputterer (E-1010; Hitachi) at 50 mtorr and 5 mA for 30 s and viewed by SEM at a voltage of 15 kV.

In vivo assessment of angiogenesis induced by HGF released from self-assembled PA nanofibers

The materials used for angiogenesis evaluation were PBS, PA aqueous solution, five doses of free HGF, and five doses of HGF with PA solution. Each experimental group consisted of 6 mice. Under anesthesia, PBS, the HGF (10 μg/mouse) solutions, and HGF with PA solution was carefully injected into the back subcutis of female BALB/c mice (6 weeks old; Shimizu Laboratory Supply, Kyoto, Japan) 1.5 cm apart from the tail root at the body center. At 1, 3, 7, 10, 14, 21, and 28 days post-treatment, the mice were sacrificed by an overdose injection of anesthetic and the skin including the injected site (2×2 cm^2) was carefully taken off for subsequent biological examinations. Photographs of the skin flaps were taken to record tissue appearance around the treated site.

Angiogenesis induced at the injection site was assessed in terms of histological and biochemical parameters. The angiogenesis of HGF was estimated by determining the amount of tissue hemoglobin as a marker of angiogenesis (11). Briefly, the tissue around the HGF injection site was scraped using a scalpel and immersed in 17 mM Tris-HCl buffer solution (pH 7.6) containing 0.75 % of ammonium chloride for 24 hr at 4 °C to extract hemoglobin from the tissue. The extracted hemoglobin was quantitated using a hemoglobin assay kit (Wako Pure Chemicals Co. Ltd., Kyoto, Japan) based on a calibration curve which had been prepared by hemoglobin standard solutions.

The granulation tissue formed around the injection site was obtained by scarping it from the skin and fascia at different time intervals after subcutaneous injection of HGF, PA, and HGF along with PA. The wet weight of the scraped tissue was measured to estimate the extent of edema or/and tissue granulation induced by HGF. In addition, of the six skin flaps, three flaps were randomly selected for histological evaluation. The skin flaps were cut at the central portion of injection site by a scalpel. One cut of the skin was fixed with 10% neutralized formalin solution, embedded in paraffin, and sectioned (2 mm in thickness), followed by staining with hematoxylin and eosin (HE). Photomicrographs of three cross sections from 3 different mice were taken at different magnifications to evaluate the vascularization histologically.

Statistical analysis

All data were statistically analyzed to express the mean ± the standard deviation (SD) of the mean. Student's *t*-test was performed and $p < 0.05$ was accepted to be statistically significant.

Results

Morphology of self-assembled PA nanofibers

Figure 1-A shows the appearance of gel formed by adding PA solution to bFGF solution. Figure 1-B shows SEM photograph of nanofibers formed through self assembly of PA. SEM photograph of self-assembled PA revealed the formation of fibrous assemblies of nanofibers with an extremely high aspect ratio, and high surface areas.

Vascularization following HGF treatment with or without PA injection

Figure 2 shows the tissue appearance of mouse subcutis 3 and 4 weeks after subcutaneous injection of free HGF, PA solution, and HGF along with PA. A transparent gel was formed immediately after injection of HGF with PA.

When HGF was injected along with PA solution, new capillaries were formed at the injected site. HGF injection alone did not contribute to vascularization, and the tissue appearance was similar to that of PA injection alone.

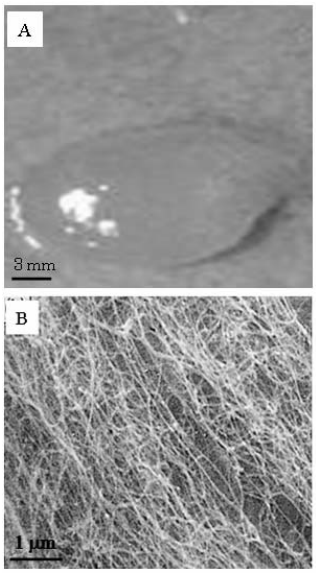

***Figure 1:** Light microscopic photographs of a gel formed by adding HGF to a PA aqueous solution (A), and SEM photographs of self-assembled PA nanofiber network (B).*

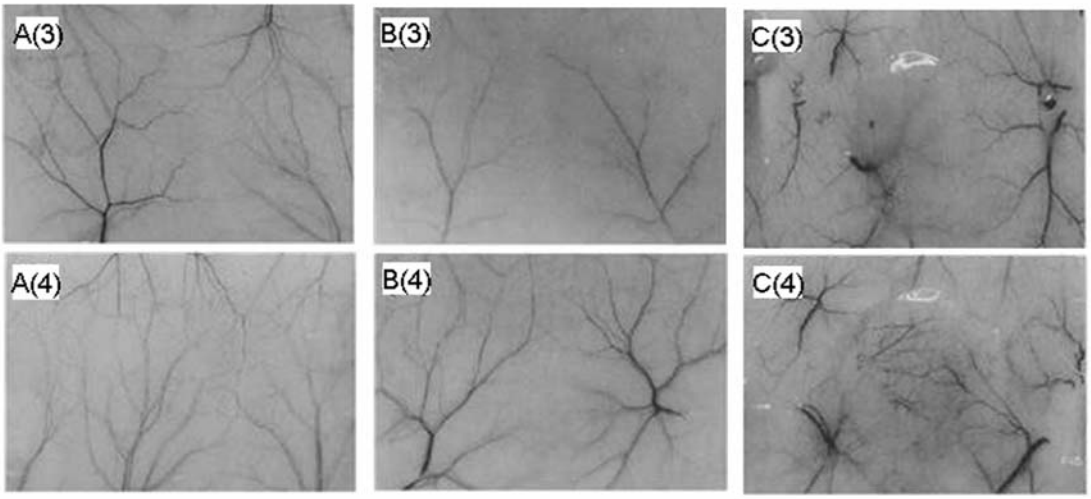

***Figure 2:** Tissue appearance of mouse subcutis 3 and 4 weeks after injection of PBS (A), free HGF (B), and HGF along with PA (C). The number in parenthesis indicates implantation time. The concentration of HGF is 10 μg/mouse.*

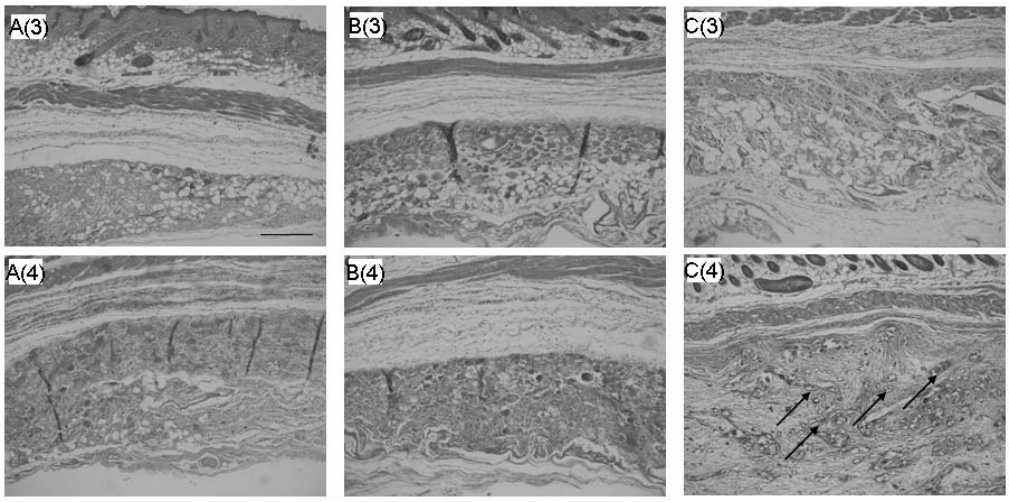

Figure 3. *Histological sections of mouse subcutis 3 and 4 weeks after injection of PBS (A), free HGF (B), and HGF along with PA (C). The number in parenthesis indicates implantation time. The concentration of HGF is 10μg/mouse. The arrows in the histological sections indicate newly formed capillaries (Magnification: × 100, HE staining).*

Figure 3 shows the histological sections of mouse subcutis 3 and 4 weeks after subcutaneous injection of free HGF, PA solution, and HGF along with PA. It is apparent that new blood vessels were formed around the subcutaneous injection site of HGF together with PA, whereas free HGF was not effective. No problem or severe inflammatory response was observed.

Discussion

Efficient vascularization of tissue-engineered scaffolds is crucial for a successful tissue engineering application. The use of angiogenic factors is a popular approach to induce neovascularization. However, growth factors have a very short half-life when injected and are unstable in solution. To overcome these problems, growth factors have been encapsulated within different types of polymeric carriers and it is conceivable to incorporate the angiogenic factor to a sustained releasing system, and using it prior to the implantation. The growth factors incorporated in these releasing systems require surgery for implantation, which is not welcomed. We report on here solid scaffolds that incorporate HGF and from aqueous solutions of peptide amphiphiles by self-assembly. The scaffold consists of nanofiber networks formed by the aggregation of the amphiphilic molecules, and this process is riggered by addition of HGF suspensions to the aqueous solutions. The scaffolds formed by these systems could be delivered to living tissues by simply injecting a liquid (i.e., peptide amphiphile solutions) and HGF solution. The injected solutions would form a solid scaffold at the injected site of tissue. The hydrogel consists of nanofiber networks formed by the aggregation of the amphiphilic molecules, and this process is triggered by addition of HGF suspensions to the aqueous solutions of peptide amphiphiles. Formation of nanofibers depends on the chemical structure of PA. PA can self-assemble into sheets, spheres, rods, disks, or channels depending on the shape, charge, and environment. Amphiphiles with a conical shape in which the hydrophilic head group is somewhat bulkier than its narrow hydrophobic tail have been shown to form cylindrical micelles (8). The PA synthesized here contains a hydrophobic domain of 16 carbons, a hydrophilic domain of AAAAGGG, and a Glu residue, following a conical shape made of RGD. The Glu residue provides a net negative charge at pH 7.4 for PA, so that the positive charge of HGF molecules can screen electrostatic repulsion among them. Thus, once electrostatic repulsions are screened by electrolytes, the molecules are driven to assemble by hydrogen bond formation and the unfavorable contact among hydrophobic segments and water molecules. Hepatocyte growth factor (HGF) has been noted as the signal molecule playing an important role in development, morphogenesis, and regeneration of living systems (3). Recently some therapeutic trials in angiogenesis induction (12, 13), chronic fibrotic diseases [14, 15], and tissue regeneration (16, 17) by HGF have been performed in the laboratory and clinically to demonstrate any potential efficacy. The most important concern regarding the delivery of proteins is whether or not the protein released in the body actually retains its biological activity. To evaluate protein activity, *in vitro* culture techniques are normally employed because of their simplicity and convenience, compared with *in vivo* animal experiments. However, any *in vitro* non-degradation system cannot be applied to evaluate the biological activity of released HGF. Thus, to obtain information on the retention of HGF activity, we directly

assessed vascularization after subcutaneous injection of PA along with HGF in animals. Figure 2 clearly indicates that subcutaneous injection of HGF along with PA was effective in enhancing HGF-induced angiogenesis. Histological examination demonstrated that vascularization was remarkable around the injection site of self-assembled PA nanofibers incorporated HGF, in contrast to sites injected with an aqueous solution of HGF. Injection of HGF in the form of a solution was not effective in inducing vascularization at all and injection of HGF-free PA alone did not induce any vascularization effect. No increase in the amount of hemoglobin was observed even when the amount of HGF in the injected solution was increased to 1 mg per mouse (data are not shown). This must be due to rapid elimination of HGF from the injection site. In contrast, the HGF incorporated in self-assembled PA nanofibers enabled us to reduce the dose that was effective in inducing significant vascularization to 10 μg per mouse. This finding strongly suggests that the HGF-incorporated self-assembled PA nanofibers still maintain their biological activities even though exposed to an *in vivo* environment. It is highly possible that the slow degradation of the HGF-incorporated self-assembled PA nanofibers causes a longer period of HGF release, resulting in a prolonged angiogenesis effect. HGF seems to be released from self-assembled PA as a result of diffusion mechanism. The enhanced vascularization is due to the sustained release of HGF.

Conclusion

These results strongly suggest that the angiogenesis in advance induced by the controlled release of HGF from HGF-incorporated PA play an important role in making an environment suitable for the survival and activity of transplanted cells. The angiogenesis would be useful for sufficient supply of nutrients and oxygen to the cells transplanted. In conclusion, prevascularization induced by the controlled release of HGF is a promising technology for tissue engineering.

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