Journal of Bioscience and Bioengineering
VOL. 118 No. 3, 333–340, 2014

www.elsevier.com/locate/jbiosc

Effect of plasma-irradiated silk fibroin in bone regeneration

Ryoichiro Uchida,[1] Ujjal K. Bhawal,[2] Hideo Kiba,[3] Kiyoshi Arai,[4] Yasuhiro Tanimoto,[1] Noboru Kuboyama,[5] Tetsuo Asakura,[6] and Norihiro Nishiyama[1,*]

Department of Dental Biomaterials, Nihon University School of Dentistry at Matsudo, 870-1 Sakaecho Nishi 2, Matsudo, Chiba 271-8587, Japan,[1] Department of Biochemistry, Nihon University School of Dentistry at Matsudo, 870-1 Sakaecho Nishi 2, Matsudo, Chiba 271-8587, Japan,[2] Department of Oral Pathology, Nihon University School of Dentistry at Matsudo, 870-1 Sakaecho Nishi 2, Matsudo, Chiba 271-8587, Japan,[3] Department of Pediatric Dentistry, Nihon University School of Dentistry at Matsudo, 870-1 Sakaecho Nishi 2, Matsudo, Chiba 271-8587, Japan,[4] University School of Dentistry at Matsudo, 870-1 Sakaecho Nishi 2, Matsudo, Chiba 271-8587, Japan,[5] and Department of Biotechnology, Tokyo University of Agriculture and Technology, Nakacho 2-24-16, Koganei, Tokyo 184-8588, Japan[6]

Received 20 November 2013; accepted 13 February 2014
Available online 26 March 2014

We have recently identified plasma-irradiated silk fibroin (P-AF) as a key regulator of bone matrix properties and composition. Bone matrix properties were tested in 48 femur critical size defects (3.25 mm in diameter) with the expression of osteoblast specific genes at 1 and 2 weeks after surgery. The scaffolds were characterized by various states of techniques; the scanning electronic microcopy revealed the large sized pores in the aqueous-based silk fibroin (A-F) scaffold and showed no alteration into the architecture by the addition of plasma irradiation. The contact angle measurements confirmed the introduction of plasma helped to change the hydrophobic nature into hydrophilic. The histological analyses confirmed the presence of silk fibroin in scaffolds and newly formed bone around the scaffolds. Immunohistochemical examination revealed the increased expression pattern in a set of osteoblast specific genes (TGF-β, TGF-β type III receptor, Runx2, type I collagen and osteocalcin). These data were the first to show that the properties of bone matrix are regulated, specifically through Runx2 pathway in P-AF group. Thus, an employment of P-AF increases several compositional properties of bone, including increased bone matrix, mineral concentration, cortical thickness, and trabecular bone volume.

[**Key words:** *Bombyx mori* silk fibroin; Scaffold; Plasma irradiation; Bone regeneration; Osteoblast]

Silk fibroin from *Bombyx mori* cocoon is a promising biomaterial that has been used for both *in vitro* and *in vivo* tissue engineering applications (1–5). The mechanical strength, biocompatibility, and slow degradation rate that facilitates material substitution with newly formed tissue, suggest that silk is suitable for use as a matrix material in bone regeneration studies (6,7). The three-dimensional porous silk fibroin scaffolds prepared in an all-aqueous format as well as an organic solvent-based (hexafluoroisopropanol: HFIP) process was reported (8,9). HFIP-based silk fibroin (HFIP-F) scaffolds are suitable for bone regeneration since the degradation rate of HFIP-F scaffold is very slow and therefore provides mechanical stability as well as support for the bone defect site until new bone formation can occur. More recently, aqueous-based silk fibroin (A-F) scaffold fabrication methods were developed, providing scaffolds with similar morphology to the HFIP-F scaffolds, but with significantly faster degradation rates (10). Furthermore, A-F scaffold preparation results in scaffolds that are more biocompatible and have been shown to provide enhanced osteogenic differentiation as compared to the HFIP-F scaffolds (11). Our group previously published a number of studies exploring the use of silk fibroin for increasing bone formation in clinically relevant fibroin-implanted rabbit femur models. Our results showed that the A-F scaffold showed higher osteoconductivity than the HFIP-F ones (12). This was due to the A-F scaffold having a more roughened wall surface than the HFIP-F scaffold, since the surface morphological characteristic strongly affected the cell proliferation, transformation of osteoblasts, and bone tissue formation as reported by Shibli et al. (13).

This report continues our characterization of scaffolds for tissue engineering applications, using plasma-irradiated three-dimensional porous A-F (P-AF) scaffolds. Plasma irradiation is a widely used method in the area of automotive (14) and biomedical devices to overcome the issue of poor wettability of polymeric materials (15). After plasma irradiation, polar groups are introduced to hydrophobic surfaces resulting in an improvement of their wettability (16). Recently, Lim et al. reported that hydrophilic surface modified by the plasma irradiation induced osteoblastic cell growth and mineral deposition and enhanced the quantity and quality of mineralization relative to hydrophobic surfaces *in vivo* (17).

The recent identification of Runx2 as a transcription factor required for bone formation was a significant milestone in osteoblast biology. Both intramembranous and endochondral ossification were blocked owing to the maturational arrest of osteoblasts in Runx2 knockout mice (18,19). We have focused on Runx2, which encodes an osteogenic transcriptional activator at the downstream end of transforming growth factor-β (TGF-β) and bone morphogenic protein (BMP) signaling pathways. The TGF-β superfamily co-receptor, the TGF-β type III receptor (TGF-β RIII, or betaglycan) is

* Corresponding author. Tel.: +81 47 360 9352; fax: +81 47 364 6295.
E-mail address: nishiyama.norihiro@nihon-u.ac.jp (N. Nishiyama).

1389-1723/$ – see front matter © 2014, The Society for Biotechnology, Japan. All rights reserved.
http://dx.doi.org/10.1016/j.jbiosc.2014.02.016

the most abundantly expressed TGF-β receptor in most cell types (20,21). TGF-β RIII is reported as a cell surface co-receptor for BMP ligands, and mediate BMP signaling in a biologically relevant assay (22,23). TGF-β RIII has classically been defined as a ligand-presenting co-receptor, promoting the binding of TGF-β super family ligands to their respective signaling receptors (24). Recent studies support the increasingly complex roles for TGF-β RIII in regulating TGF-β receptor trafficking (25–27) and both Smad-dependent (25,28) and Smad-independent signaling (28,29).

Appropriate vascularization is emerging as a prerequisite for bone development and regeneration, and indeed there appeared to be a developmental reciprocity between scaffolds and osteoblasts. This study investigated the biocompatibility and biological effects of P-AF on osteoblast cells, with the aim of optimizing silk scaffolds based bone defect repair. The extent of osteoconductivity was assessed by histology for bone-related outcomes, along with bone mineral content (BMC), bone volume/total volume (BV/TV). The enhancement of osteoblast specific genes at P-AF implantation in the rabbit femur might be important in bone regeneration and improved bone healing. Our results indicate that P-AF-induced Runx2 regulation plays a key role in mediating the cross-talks and balances between bone markers and Runx2 signal. Thus, it has been proposed that P-AF may act as organizing sites, where proteins involved in signal transduction are compartmentalized and brought into proximity with cell-surface signaling receptors.

MATERIALS AND METHODS

All chemical reagents are purchased from Wako Pure Chemical Industries, Osaka, Japan, unless otherwise indicated.

Preparation of freeze-dried silk fibroin Preparation of silk fibroin was described previously (30). In brief, *B. mori* cocoons were heated in deionized water at 95°C for 20 min and the threads were then reeled. The air-dried threads were degummed in a solution containing 0.08% (w/v) sodium carbonate and 0.12% (w/v) Marseille soap for 120 min at 95°C. The degummed silk fibers were dissolved in calcium chloride ($CaCl_2$)–water–ethanol (molar ratio = 1:8:2) solution at 70°C for 1 h and then dialyzed against Milli-Q water for 3 days at 4°C using a cellulose membrane (MWCO: 14,000; Viskase, Darien, IL, USA). The dilute silk fibroin was then rapidly frozen and freeze-dried.

Preparation of silk fibroin sponge Preparation of silk fibroin sponge was described previously (30). In brief, for the aqueous-based fibroin (A-F) scaffold, the 4% Milli-Q aqueous solution of silk fibroin was poured into a 3 ml syringe (volume: 0.5 ml; internal diameter 3.25 mm), which had been tightly packed with sodium chloride grains (grain size: above 500 μm). The sodium chloride grains were then leached out with Milli-Q water and subsequent freeze drying.

The HFIP-based fibroin (HFIP-F) scaffold was prepared by pouring the 4% HFIP solution of silk fibroin into the syringe, which had been tightly packed with sucrose grains (grain size: above 500 μm). All parts of the syringe were resistant to the HFIP. After allowing the HFIP to evaporate at room temperature, the fibroin was insolubilized by treatment with absolute methanol. The sucrose was then leached out using Milli-Q water. Methanol was removed from the resulting porous sponge by washing in many changes of Milli-Q water at 4°C for a few days and subsequent freeze drying.

Next, the 3 mm diameter cylindrical fibroin sponge scaffolds were cut transversely into 3 mm-height discs, which were sterilized dry with ethylene oxide gas at room temperature before implantation.

Plasma treatment Plasma treatment was performed with a radiofrequency (RF) corona discharge apparatus (Plasmastream PSC1001, Peal Kogyo, Inc., Osaka, Japan) to make P-AF scaffold from A-F scaffold. It consisted of a plasma nozzle and a sample stage. It was operated with a frequency of applied voltage of 16 kHz with an input power of 30 W. Air gas flowed through the nozzle (5 L/min), and a high RF-voltage (7 kV) was coupled to the electrode. The length of the plasma nozzle was located 20 mm above the A-F scaffold surface on the sample stage. The A-F scaffold keep a little away from the plasma plume tip (end of discharge) avoid to carbonization of the surface. The A-F was plasma treated on both top and bottom sides for 1 min in the atmosphere.

Measurement of contact angles Surface contact angle measurements were assess the change in surface hydrophobicity of the silk fibroin scaffold after plasma irradiation. Static contact angle measurements were performed using the sessile drop method on a goniometer (Dropmaster MD100, Kyowa Electronic Instrument Co., Ltd., Tokyo, Japan) under ambient conditions at room temperature. Pure water (1 μL) was dropped on the surfaces and the contact angle was immediately measured after water drop on the surface. The A-F, P-AF and HFIP-F samples of each surface type were measured ($n = 5$).

Surgical procedure A total of 24 male Japanese white rabbits (12 weeks old) were used. The rabbits were allowed for free access to food and water ad libitum, and they were maintained on a 12 h light/dark cycle (lights on 8:00 to 20:00 h) at 23 ± 1°C, humidity 60 ± 10% for a period of 1 week before surgery. The animals were randomly divided into four groups: group 1 (control: no implanted group), group 2 (HFIP-F implant group), group 3 (A-F implant group) and group 4 (P-AF implant group). All animals were treated and maintained according to the Japanese Laboratory Regulations. The study was approved by the Animal Care and Use Committee of Nihon University School of Dentistry at Matsudo, Chiba, Japan (08-0034).

All surgical procedures for implantation into the lateral epicondyle of the rabbit femur were performed under general anesthesia. For induction, a single dose of 17.5 mg/kg bodyweight of sodium thiopental (Tanabe Seiyaku, Osaka, Japan) was administered intravenously. Anesthesia was maintained with 2% isoflurane (Dainippon Pharmaceutical Co., Osaka, Japan) given continuously with a mask at an oxygen flow rate of 1.5 L/min. A 2 cm-longitudinal incision was made lateral to the femur, the lateral epicondyle was exposed, and the periosteum was retracted. A pilot hole was prepared with a round bur (ISO 014; Dentsply, PA, USA) using a dental hand engine (Nobel Biocare AB, Goteborg, Sweden), and then gradually widened using fisherburs (ISO 023; Dentsply) with a low rotational drilling speed under sterile saline irrigation. The hole was drilled with an implant drill (Driva TMDrills, diameter: 3.25 mm; Zimmer, CF, USA) to a depth of 4.95 mm under sterile saline irrigation to fit the scaffold size (3 × 3 mm). After scaffold was implanted into the bone defect, the soft tissues were closed in separate layers using absorbable sutures. At 1 week or 2 weeks after implantation, the rabbits were sacrificed using carbon oxide gas and the femur epicondyle bone with artificial bone defect and attached muscles was extirpated. The extirpated tissues were immersion fixed in 4% w/v formaldehyde in 0.1 M phosphate buffer (pH 7.4) for at least 48 h at 4°C.

For micro-CT analysis, wax histology and immunohistochemistry, tissue blocks containing the implantation site and surrounding bone were prepared using a micro cutting machine (EXAKT BS300CL; Meiwa, Tokyo, Japan).

Micro-CT scanning and image segmentation The micro-CT image of the fixed femur epicondylar bone was observed using an *in vivo* micro-CT system (I-View@ software; J. Morita, Kyoto, Japan) with an X-ray source of 90 kV/88 μA, over a full 360° rotation, and an exposure time of 2 min. The isotropic resolution was 30 × 30 × 30 μm voxels. The imaging of the artificial bone defect of each sample was then carried out in the same way. Based on the original CT images of each sample, three-dimensional (3D) images were reconstructed using I-View@ software.

Micro-CT analysis of new bone formation inside the artificial bone defect The 3D image data for each sample was calibrated by a hydroxyapatite (HA) phantom provided by the system manufacture in conjunction with a morphometric program (TRI/3D-BON; Ratoc System Engineering, Tokyo, Japan). This phantom consisted of five HA phantoms of known density (100, 200, 400, 600, and 800 mg HA/ml). The bone volume (BV) and bone mineral content (BMC) of the new bone, which had formed inside of the scaffold implanted within the artificial bone defect were then determined. For BMC, 3D bone mineral density (BMD) color images of the artificial bone defect were prepared at 1 and 2 weeks after surgery. The defect depths were measured in the center of the scaffold.

Conventional wax histology The fixed tissue samples were decalcified with an aqueous solution containing 10% v/v formic acid, 4% formaldehyde, and 0.1 M Sorensen's phosphate buffer. The decalcified tissues were embedded in paraffin and serial 4 μm sections were cut parallel to the longitudinal axis of the fibroin implant and stained with hematoxylin and eosin, and examined and micrographed using bright field light microscopy.

Immunohistochemical examination Formalin-fixed, paraffin-embedded specimens were subjected to antigen retrieval, endogenous peroxidase blocking and rinsed with phosphate-buffered saline (PBS). Immunostaining was performed using CSA system (Dako, Carpinteria, CA, USA). Sections were initially immersed in Target Retrieval Solution (Dako) at 97°C for 40 min, and subsequent steps were performed according to manufacturer's instructions. Endogenous peroxidase activity was blocked by a reagent (Dako Real Peroxidase-Blocking Solution; S2023) for 30 min. Antibodies against Runx2, TypeIcollagen, osteocalcin, TGF-β and TGF-β RIII, were used as primary antibodies. The secondary antibody was incubated at room temperature for 30 min. The immunostaining of all specimens was performed simultaneously to ensure the same antibody reaction and DAB exposure conditions.

Statistical analysis All results were expressed as mean ± SD. Micro-CT and contact angle data were analyzed using one-way ANOVA followed by post hoc tests for pair wise comparisons (Tukey's test). Differences were considered to be significant for $p < 0.05$.

RESULTS

Morphological observation of fibroin sponge as a scaffold The HFIP-F scaffolds showed a porous structure with approximately 500 μm in diameter of which had been developed by removing sucrose crystals with water rinsing (Fig. 1). These cavities