Synthetic Osteopontin-derived Peptide SVVYGLR can Induce Neovascularization in Artificial Bone Marrow Scaffold Biomaterials

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Received October 31, 2006/Accepted February 16, 2007

We have previously reported that an osteopontin-derived SVVYGLR peptide exhibited potent angiogenic activity in vitro and in vivo. In the present study, the focus points were on the in vitro effect of SVVYGLR on bone marrow stromal cell proliferation, as well as its in vivo effect on bone tissue formation when grafts made of CO3:Ap-collagen sponge — as a scaffold biomaterial containing the SVVYGLR motif — were implanted. SVVYGLR peptide promoted bone marrow stromal cell proliferation. When a CO3:Ap-collagen sponge containing SVVYGLR peptide was implanted as a graft into a tissue defect created in rat tibia, the migration of numerous vascular endothelial cells — as well as prominent angiogenesis — inside the graft could be detected after one week. These results thus suggested that our scaffold biomaterials including the peptide could be useful for bone tissue regeneration.

Keywords: CO3:Ap-collagen, Bone marrow scaffold biomaterials, SVVYGLR

INTRODUCTION

In many western countries, aging societies are on a rapid increase. With many elderly people suffering from diverse bone diseases, development of regenerative bone tissues is a fast-growing sector that is highly required.

Recent progress in tissue engineering has led to a focus on hard tissue biomaterials. On this note, various composite scaffold biomaterials have been investigated from the viewpoint of biodegradability. In particular, CO3:Ap-collagen composites have been shown to be successful, chiefly because CO3:apatite (CO3:Ap) displays similar crystallinity and chemical composition to bone3. Indeed, CO3:Ap-collagen composites have shown good biocompatibility when implanted into the abdominal wall and beneath the periosteum cranii of rats. However, these composites had no space in the inner bulk for cell invasion. Against this background, CO3:Ap-collagen sponges were studied5.

CO3:Ap-collagen sponges with larger pores induced more proliferation and invasion of osteoblasts. Furthermore, growth factors such as BMP (Bone Morphogenetic Protein)-2 and BMP-7 promoted marked bone formation using bone repair biomaterials5. However, it must be highlighted that neovascularization — a process to supply oxygen and nutrients — is also essential for tissue regeneration and sufficient cell function.

The process of angiogenesis starts from digestion of the basement membranes of blood vessels by endothelial cells5. Cells subsequently migrate, proliferate, and form tube-like structures. Numerous researchers have reported that these cellular responses are tightly regulated by signals from various growth factors and cytokines, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and interleukin-856. Osteopontin, one of the extracellular matrix proteins, is a phosphoric acid protein containing a large quantity of sialic acid. This protein is widely distributed in various areas such as bone tissue, kidney, brain, and skin. Osteopontin participates in bone metabolism and also mediates inflammatory responses and angiogenesis6.

Recently, a novel binding sequence, Ser-Val-Val-Tyr-Gly-Leu-Arg (SVVYGLR), has been identified as an amino acid sequence in OPN involved in angiogenesis89. This motif might be important in pathological conditions as SVVYGLR, adjacent to the RGD sequence and hidden in the whole osteopontin molecule, is exposed by protease cleavage.

To date, we have already succeeded in artificially synthesizing SVVYGLR peptide as an angiogenic factor10. Based on the finding that OPN is widely distributed in osseous tissue, we sought to
investigate the effects of SVVYGLR on bone marrow-derived mesenchymal stem cells and osseous tissue in the present study. To this end, cell proliferation assay was conducted using bone marrow stromal cells. Besides, to assess the effects of SVVYGLR on bone formation, an animal study using grafts made of CO₃Ap-collagen sponge as a scaffold biomaterial containing the SVVYGLR motif was also carried out.

MATERIALS AND METHODS

Synthesis of CO₃Ap
Carbonate-containing apatite (CO₃Ap) was synthesized at 60 °C and pH 7.4 ± 0.2. A 0.5-L solution of 100 mmol/L Ca(CH₃COO)₂ • H₂O, and a 0.5-L solution of 60 mmol/L NH₄HPO₄ containing 60 mmol/L (NH₄)₂CO₃, were added into a mechanically stirred solution of 1.3 mol/L acetate buffer. The suspension was stirred for three hours, then kept at 25 °C for 24 hours. CO₃Ap was separated by filtration, washed with distilled water, and dried at 60 °C.

Preparation of CO₃Ap-collagen sponges
First, 0.5 wt% of Cellgen calf skin collagen solution (Koken, Tokyo, Japan) that had been treated using enzymes to minimize antigenicity was neutralized with 0.05 N NaOH, then mixed immediately with 70% (w/w) CO₃Ap by dry weight. Gel portions were put into a 96-well plate, which was then frozen at -80 °C for two hours and dried in a freeze-drying machine (Eyela, Tokyo, Japan) for 24 hours. Samples were subjected to ultraviolet (UV) radiation for four hours, at 10 cm from the UV lamp (10 W, 253.7 nm), to make the samples insoluble. In a typical 70% (w/w) CO₃Ap-collagen sponge prepared by lyophilizing, pores of approximately 50 - 300 μm in size appeared to continue deep into the sponge. Coagulated CO₃Ap crystals were observed with the fibrous ribbons of collagen in the CO₃Ap-collagen sponge.

Synthesis of SVVYGLR peptide
SVVYGLR was synthesized on PEG-PS graft copolymer beads (the latter as support) with a high-performance solid phase method using a PSSM-8 automatic peptide synthesizer (Shimadzu, Kyoto, Japan). After assembly of the peptide chain, side chain protection was removed to leave a resin-bound peptide. The peptide was characterized by high performance liquid chromatography (Shimadzu, Kyoto, Japan). Mass value of the peak matched the theoretical mass value of 792.616 for SVVYGLR, indicating that the synthetic product represented pure SVVYGLR peptide. CO₃Ap-collagen sponges were immersed in 100 ng/ml of SVVYGLR peptide solution on a clean bench overnight.

Bone marrow-derived cells
Rat bone marrow stromal cells (rBMSCs) were obtained by a protocol approved by the Animal Research Subjects Committee of Osaka University Graduate School of Dentistry (No.05-019). Bone marrow cells were collected from femurs of eight-week-old male Sprague-Dawley rats as described previously and maintained in a growth medium consisting of alpha-modified minimum essential medium (α-MEM) supplemented with 15% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Nacalai Tesque, Kyoto, Japan) at 37 °C with 5% CO₂. Non-adherent cell population was removed after 24 hours, and the adherent rBMSC layer was washed twice with fresh medium. Cells were then continuously cultured, and passages were performed when cells were in subconfluent state.

Cell proliferation assay
The effect of SVVYGLR on rBMSC proliferation was assessed by WST-1 assay using a commercially available measurement kit (Cell Counting Kit; Dojindo Laboratories, Kumamoto, Japan) as described previously. rBMSCs were seeded in 96-well tissue culture plate (4 × 10⁴ cells per well) and maintained in the growth medium. After 24 hours’ incubation, culture medium was changed with a fresh growth medium containing 1, 10, 100, or 1000 ng/ml of SVVYGLR. Cells were continuously cultured, and the culture medium containing SVVYGLR was renewed every other day. The number of rBMSCs was evaluated after 0, 4, 5, 6, and 7 days as follows: 10 ml of the working solution containing WST-1 was added to each well, and then cells were incubated for two hours at 37 °C with 5% CO₂. Absorbance of each well was measured by a MTP-32 microplate reader (Corona Electric, Ibaraki, Japan) at 415 nm.

Pellet culture
Pellet culture was basically carried out as described previously. A total of 5 × 10⁴ rBMSCs were pelleted by centrifugation at 300 × g for two minutes in a 2-ml microtube (MultiDolphin, Nippon Genetics, Tokyo, Japan) in 1 ml of α-MEM (high glucose) supplemented with 100 nM dexamethasone, 50 ng/ml ascorbic acid (Sigma-Aldrich, St. Louis, USA), ITS+ Premix (BD Biosciences, Bedford, USA; final concentrations: 6.25 ng/ml insulin, 6.25 ng/ml transferrin, 6.25 ng/ml selenious acid, 5.35 μg/ml linoleic acid, 1.25 mg/ml bovine serum albumin), 10 ng/ml recombinant human transforming growth factor-β1 (PeproTech EC, London, UK), and with or without 100 ng/ml of synthesized peptide SVVYGLR. Cells...
were continuously cultured for 26 days with fresh medium change every other day.

Pellets were harvested and fixed in 10% phosphate-buffered formalin (Wako, Osaka, Japan) for three hours at 4°C. After washing with phosphate-buffered saline (PBS, pH 7.4), the pellet was immersed in PBS in a cell culture plate, and a projective picture of the pellet was taken under phase contrast microscopy at 40X 40 magnification. The total area of the pellet image was analyzed by ImageJ software version 1.33u (National Institutes of Health, Bethesda, USA).

Animal experiment
Study protocol of this experiment was approved by the Institutional Animal Study Committee. Subjects comprised four-week-old SPF/VAF Crl : CD(SD) male rats (Nihon Animal, Osaka, Japan). To the rats, 50 mg/kg of pentobarbital was administered, and UV-irradiated CO3Ap-collagen sponge containing the SVVYGLR peptide was surgically embedded into the bone marrow of the tibia. A drill was used to make a 3Ø 7-mm hole on the tibia without exposing the bone marrow, and a bone chisel was used to expose bone marrow. A single layer of bone marrow was scraped off to create a space for graft placement. Animals were killed one and two weeks postoperatively, and implants were dissected with surrounding tissue. Specimens were immediately embedded in OCT Compound (Tissue Tek, CA, USA) in acetone-dry ice.

Immunohistochemistry
Polyclonal antibody to factor VIII (von Willebrand, Dako, Denmark) was used as the primary antibody. Cryosections, 6 Øm in thickness, were fixed in 10% buffered formalin for 10 minutes and incubated with primary antibody at a dilution of 1 : 400 for 14 hours at 4°C. After rinsing in Tris-HCl buffer containing 0.1% Tween-20, the sections were treated with biotinylated anti-rabbit immunoglobulin (Amersham, NJ, USA) for 30 minutes at room temperature, rinsed, and then incubated with alkaline phosphatase-labeled streptavidin (Dako, Glostrup, Denmark). Alkaline phosphatase activity was visualized in new fuchsin. Nuclear details were enhanced by counterstaining with hematoxylin. In addition, cryosections were stained with hematoxylin and eosin (H&E).

Statistical analysis
Quantitative tests were carried out in quadruplicate, and mean values with standard deviations were calculated. Statistical analysis of data was accomplished by one-factor analysis of variance. Dunnett’s multiple comparison test was used for comparison at 99% confidence interval.

RESULTS

A WST-1 cell proliferation test was conducted to investigate proliferation of rBMSCs in vitro (Fig. 1). At 4th to 7th day after exposure to peptide, cell proliferation for 100 and 1000 ng/ml of SVVYGLR was significantly higher than the control (0 ng/ml SVVYGLR). These findings indicated that SVVYGLR facilitated initial proliferation of rBMSCs in vitro.

Growth of bone marrow stromal cells was investigated ex vivo using a pellet culture test (Fig. 2). Cells were cultured with 100 ng/ml of SVVYGLR for 26 days. Compared to the control (0 ng/ml SVVYGLR), pellets with SVVYGLR displayed a 1.5-fold increase in size. Tissue sections were stained using toluidine blue, and enlargement of the pellets was caused by the proliferation of bone marrow stromal cells, rather than due to extracellular matrix (data not shown). These findings suggested that SVVYGLR facilitated growth of rBMSCs in a long-term pellet culture.

A CO3Ap-collagen sponge containing the synthetic motif, SVVYGLR, was implanted as a graft into a tissue defect in rat tibia. Histological analysis showed marked migration of vascular endothelial cells inside the graft at one week after implantation (Fig. 3B). The presence of vessels could be estimated by immunostaining for factor VIII specific for vascular endothelial cells. Blood vessels were clearly seen in the boundary region between bone marrow and the graft made of CO3Ap-collagen sponge (Fig. 3B). After one week, the migration of numerous factor VIII-positive vascular endothelial cells toward the porous internal structure of CO3Ap-collagen sponge was observed. Numerous bone marrow stromal cells were also seen in the porous internal structure of the CO3Ap-collagen sponge. In trabecular bone, a Haversian canal made of factor VIII-positive vascular endothelial cells was found (Fig. 3A).

On the other hand, CO3Ap-collagen sponge without the SVVYGLR motif did not induce factor VIII-positive fragments (Fig. 3C), and vessels were not even detected in the boundary region between the bone marrow and graft. Furthermore, bone marrow stromal cells were hardly found in the porous internal structure of the graft.

Then, be it implantation with or without the synthetic SVVYGLR motif, CO3Ap-collagen sponge did not disappear, was neither absorbed nor decomposed, but remained porous, after one week.
Fig. 1  Effects of SVVYGLR on rBMSC proliferation. rBMSCs were seeded in 96-well tissue culture plates and cultured in medium containing 1, 10, 100, or 1000 ng/ml of SVVYGLR or medium alone. Number of rBMSCs was evaluated at the indicated time points by WST-1 assay. Data are mean values ± SD, n=6 for each condition. *: P<0.01, #: P<0.05, analysis of variance followed by Dunnett’s multiple comparison test to compare against the control group (medium alone).

Fig. 2  Effect of SVVYGLR on rBMSC proliferation in pellet culture. rBMSCs were cultured as a pellet for 26 days in pellet culture medium with 100 ng/ml of SVVYGLR or pellet culture medium alone. A projective picture was taken under phase contrast microscopy (insets: bars at 100 μm), and total area of the pellet image was measured by image analyzing software. Data are mean values ± SD, n=3 for each condition. *: P<0.01, analysis of variance followed by Dunnett’s multiple comparison test.

Fig. 3  Immunostaining of factor VIII in normal trabecular bone tissue (panel A) and regenerative bone tissue in CO3Ap-collagen sponge with (panel B) and without (panel C) SVVYGLR peptide. Arrow: Factor VIII-positive cells; Arrowhead: CO3Ap-collagen sponge; #: Bone marrow.
DISCUSSION

Since the end of the 20th Century, tissue engineering has witnessed leaps and bounds in its research and development. Despite the rapid progress, more excellent scaffold biomaterials await us in the near future. To date, numerous scaffold materials for bone formation have been studied since bone is one of the most important target tissues for regenerative medicine. The major focus of tissue engineering research is not only on high-quality hard tissue biomaterials, but also effective cell influx into scaffold materials to generate substantial bone tissue. Ohgushi et al. reported that osteoblasts could invade into porous hydroxyapatite with a pore size of several hundreds of micrometers. As osteoblast invasion requires a porous structure, we successfully created a 70% (w/w) CO3Ap-collagen sponge using pores of 50 - 300 μm.

Extracellular matrix components such as collagen, together with apatite crystals, may contribute to bone morphology and function with cell-matrix and matrix-matrix interactions. Pertaining to the space in which the extracellular matrix interacts, porosity and pore size are important factors. Considering that bone tissue contains approximately 60 - 70% (w/w) CO3Ap, 70% (w/w) CO3Ap-collagen sponge is thus suitable for therapeutic use, and hence prepared in this study.

As shown in our results, CO3Ap-collagen composites have shown suitable biocompatibility and CO3Ap-collagen sponges provide sufficient space for cells to invade. However, even if cells can enter the sponge biomaterial, the supply of oxygen and nutrients is crucial for cell viability and function. On this note, various scaffold materials for hard tissue reconstruction and regeneration have been investigated. Some materials have shown good promotion of bone formation. However, to obtain better bone formation, neovascularization is imperative. Against this background, following the development of scaffold biomaterials that promoted bone formation, we further synthesized SVVYGLR peptide which shows potent angiogenic activity with CO3Ap-collagen sponge.

Angiogenesis plays a key role in a variety of physiological and pathological conditions. Regulation of angiogenesis is thus expected to facilitate cure or improvement in numerous diseases. Angiogenesis involves multiple steps, such as basement membrane degradation, endothelial cell migration, proliferation, tube formation, and blood vessel maturation. The exact mechanisms involved in this process are poorly understood, but it suffices to know that both cell-matrix interaction and the effect of matrix-bound growth factors play pivotal roles.

We found a small peptide, SVVYGLR, inside the osteopontin matrix protein, and which might have a much stronger angiogenic activity than VEGF. Previous in vitro studies have indicated that SVVYGLR could induce tube formation of endothelial cells as well as binding and migratory activities of endothelial cells, but no proliferative activity was present. The present in vitro and ex vivo results demonstrated that SVVYGLR facilitated proliferation of bone marrow stromal cells. In our in vivo study, SVVYGLR clearly recruited vascular endothelial cells in CO3Ap-collagen sponge biomaterial and facilitated proliferation of bone marrow stromal cells in the graft.

Small peptides such as SVVYGLR provide several advantages: fast metabolism; low risk of immune response; and low cost due to low molecular weight. In this study, it was shown that CO3Ap-collagen sponges combined with SVVYGLR seemed suitable as a scaffold biomaterial, serving as a proper environment for angiogenic activity of endothelial cells and facilitating proliferation of bone marrow stromal cells. In particular, CO3Ap helps a sponge to maintain a stable shape against certain compressive force. In this manner, CO3Ap acts as a source of bone, differing from pure collagen sponge. CO3Ap-collagen sponges can also be implanted with press-fit technique, which is advantageous for hemostasis.

At one week after implantation, the CO3Ap-collagen sponge remained porous without showing absorption or decomposition, which is advantageous for tissue regeneration. Since rapid graft absorption and decomposition result in dead space in the tissue, rates of graft absorption and decomposition are important factors for biomaterials. In the present study, the porous structure of the graft was maintained without decomposition, providing a good scaffold for angiogenesis and bone marrow stromal cell proliferation. However, it must be mentioned that angiogenesis and bone marrow stromal cell proliferation were not seen in the control graft. In other words, the porous structure itself did not directly induce these biological phenomena, but was clearly related indirectly to these phenomena by serving as a good scaffold.

Although our results showed the limited effects of SVVYGLR on proliferation of rBMSCs, this synthetic peptide indeed affected BMSC proliferation. Thus, our herein-developed scaffold biomaterial possibly regenerated osseous tissue by the dual effects of SVVYGLR on BMSC proliferation and on angiogenic activity of endothelial cells in bone marrow. In conclusion, results of the present study suggested that CO3Ap-collagen sponge containing the synthetic motif SVVYGLR might represent a potent tissue engineering material for the treatment of diverse bone diseases.
REFERENCES


