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Enhanced bone regeneration via multimodal actions of synthetic peptide SVVYGLR on osteoprogenitors and osteoclasts

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ABSTRACT

Recently, the binding sequence Ser-Val-Val-Tyr-Gly-Leu-Arg (SVVYGLR) was found adjacent to the RGD sequence in osteopontin, suggesting involvement in osteo-immune cross-talk. The aim of this study was to investigate bioactive functions of a synthetic SVVYGLR peptide in osteoprogenitor cells and osteoclasts, and to examine potential applications in bone regeneration. The SVVYGLR peptide significantly enhanced the adhesion and proliferation of several human mesenchymal cells including bone marrow-derived mesenchymal stem cells, and $\alpha v\beta 3$ integrin was involved in cell attachment to the peptide. Additionally, the peptide reduced the number of TRAP-positive multinucleated cells during osteoclastogenesis of RAW264.7 cells and normal murine pre-osteoclasts, and also suppressed NFAT activity and expression of osteoclastogenesis-related mRNAs. When standardized bone defects in rat calvariae were filled with a collagen sponge containing the peptide or PBS (control), the number of TRAP-positive osteoclasts in the grafted sites after 3 weeks was significantly lower in the peptide group. By the 5th week, significantly enhanced resorption of the grafted collagen sponge and new bone formation was observed within and surrounding the sponge in the peptide group. These data suggest that SVVYGLR is an effective bioactive peptide for bone tissue regeneration that promotes attachment and proliferation of osteogenic cells while also suppressing osteoclastogenesis.

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1. Introduction

Bone tissue engineering is an emerging biomedical field that applies the principles of biology and engineering to the development of viable bone substitutes that restore and maintain the function of human tissues [1,2]. Bone regeneration has been facilitated by the advent of tissue engineering technologies such grafted scaffolds releasing bone morphogenetic protein-2 (BMP-2) [3,4] and basic fibroblast growth factor (bFGF) [5] proteins. These growth factors have great potential for tissue engineering and regenerative medicine; however, there are associated disadvantages including immunogenicity, relatively high cost, large molecular weight (100–200 amino acids), instability *in vivo*, and difficulty in sterilization [6]. Another approach to the release of bioactive factors uses small peptides containing only the cell-binding sequence of natural protein ligands. Small peptides are advantageous due to their ease of synthesis and handling, as well as their low immunogenic activity.

Numerous reports have described biological activity of matrix-derived synthetic peptides corresponding to active sites in proteins [6–8]. Recently, a binding sequence Ser-Val-Val-Tyr-Gly-Leu-Arg (SVVYGLR) was found adjacent to the Arg-Gly-Asp (RGD) sequence in osteopontin following thrombin cleavage at Arg 168 (Fig. 1A) [9]. This cryptic SVVYGLR motif binds the integrins $\alpha 4\beta 1$ [10], $\alpha 4\beta 7$ [11], and $\alpha 9\beta 1$ [9]. Although they only contain seven amino acids, synthetic SVVYGLR peptides have been shown to activate *in vitro* adhesion, migration and tube formation of endothelial cells [12]. Angiogenesis upon synthetic SVVYGLR peptide treatment was confirmed *in vivo* and a key tyrosine (Y) was identified for this behavior [13]. Synthetic SVVYGLR peptide was also shown to promote neovascularization in a carbonate apatite-collagen sponge graft in rat tibia bone marrow [14]. Bone is a highly vascularized

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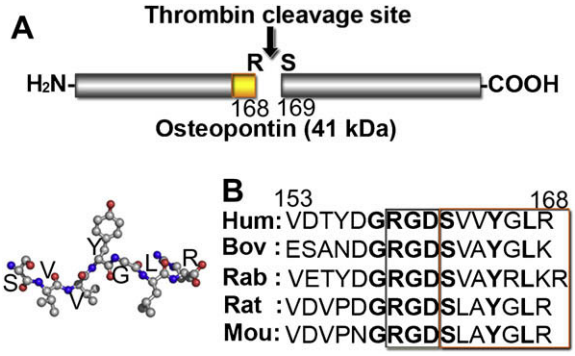

Fig. 1. (A) Schematic diagram showing the exposed SVVYGLR sequence (orange square) at a thrombin cleavage site within human osteopontin (above) with the corresponding 3D structure of the peptide (below). (B) Alignment of the sequences around the RGD site in human, bovine, rabbit, rat, and mouse osteopontin. The cryptic epitope is enclosed by the orange square.

tissue and angiogenesis is a key process in bone regeneration and bone engineering [15]. These findings therefore imply that the small synthetic peptide SVVYGLR might be a potentially useful bioactive material for bone tissue engineering.

Osteopontin, an extracellular matrix protein widely distributed in osseous tissue, participates in bone metabolism and also mediates inflammatory responses and angiogenesis [16,17]. Recently, osteopontin signaling via the SVVYGLR motif was shown to play an important role in pro-matrix metalloproteinase 9 activation in aortic mesenchymal cells [18]. The SVVYGLR sequence in human osteopontin is replaced by SLAYGLR in rat and mouse osteopontin (Fig. 1B). Recent studies demonstrated the critical involvement of SLVYGLR and SVVYGLR sequences in the pathogenesis of murine and monkey models of rheumatoid arthritis [19,20], therefore implying that the SVVYGLR sequence affects osteo-immune cross-talk.

These findings and the original location of SVVYGLR in osteopontin led us to hypothesize that the integrin-mediated interactions of synthetic SVVYGLR peptides with osteoblast and/or osteoclast precursor cells could affect bone metabolism and promote bone regeneration. The aim of this study was to investigate the bioactive functions of synthetic SVVYGLR in osteoprogenitor cells and osteoclasts, and to examine potential applications in bone regeneration.

2. Materials and methods

2.1. Synthesis of SVVYGLR peptide

SVVYGLR peptide was synthesized on PEG-PS graft copolymer beads as a support with a high-performance solid-phase method using a PSSM-8 automatic peptide synthesizer (Shimadzu, Kyoto, Japan). After assembly of the peptide chain, the side chain protection was removed to leave a resin-bound peptide. This peptide was characterized by high-performance liquid chromatography (Shimadzu). The mass value of the peak matched the theoretical mass value of 792.616 for SVVYGLR. This indicated that the synthetic product was pure SVVYGLR peptide.

2.2. Cell culture

Human bone marrow-derived mesenchymal stromal stem cells (*hMSCs*: Riken Bioresource Center, Tsukuba, Japan) from normal human ilium bone marrow (MSC-R36: Japanese male, 30 years old) were graciously supplied by Prof. Yukio Kato (Hiroshima University) and maintained in Dulbecco's modified Eagle medium (DMEM: Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS: JRH Biosciences, Lenexa, KS), 3 ng/ml bFGF (Peprotech, London, UK), 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Nacalai Tesque) in accordance with the supplier's instructions [21]. The hMSCs used in this study were between passage numbers of three and five.

Human periodontal ligament fibroblasts (*hPLFs*) from normal human periodontal ligament tissue were purchased from Lonza Walkersville (Basel, Switzerland) and maintained in Minimum Essential Medium Eagle, Alpha Modification (α-MEM: Nacalai Tesque) supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B.

Primary human gingival fibroblast (*hGF*) cultures were established from discarded healthy gingival tissues at surgery with donors' informed consent [22]. Briefly, the gingival tissue specimens were treated overnight with 0.025% trypsin and 0.02% EDTA at 4 °C. After trypsin neutralization, the lamina propria mucosae separated from the epithelial layer were minced into pieces in a plastic tissue culture dish and maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. When fibroblasts migrated out of the tissue, tissues were removed and cells were cultured until they reached confluence.

Human umbilical vain endothelial cells (*HUVECs*) were purchased from Lonza (Basel, Switzerland) and maintained in commercial Endothelial Growth Medium-2 (EGM-2: Lonza Walkersville).

Murine macrophage-like RAW264.7 cells were provided from Riken Bioresource Center and maintained in α-MEM medium (Sigma, St. Louis, MO) containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. To monitor the activity of nuclear factor of activated T-cells (NFAT) in RAW264.7 cells, cells were transfected with the NFAT response element/luciferase reporter vector (pGL4.30. Promega, Madison, WI) using TransFast™ transfection reagent (Promega) and selected using hygromycin. Of the hygromycin-resistant cells, a clone that stably luminesced upon osteoclastogenic stimulation by RANKL was selected (pNFAT/Luc-RAW264.7 cells) for the NFAT reporter assay.

Murine bone marrow monocyte/macrophage (*BMM*) lineage progenitors were collected from the femur and tibia of 8-week-old male ddY strain mice and cultured in α-MEM medium supplemented with 2 mM L-glutamine (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin, 50 ng/ml human recombinant macrophage-colony stimulating factor (M-CSF: Leukoprol, Kyowa Hakko, Tokyo, Japan), and 10% FBS [23].

Chinese hamster ovary (CHO)-K1 cells and human integrin αvβ3-overexpressing CHO-K1 cells (αvβ3-CHO) [24] were maintained in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

2.3. In vitro osteogenesis

For osteogenic induction of hMSCs and hPLFs, cells were cultured in growth maintenance medium supplemented with 0.1 μM dexamethasone, 10 mM β-glycerophosphate and 50 μM ascorbate-2-phosphate (Sigma) [25]. To demonstrate osteogenesis, a standard alkaline phosphatase (ALP) assay and von Kossa staining were used as previously described [26]. The final image of von Kossa staining was digitized, and positively stained areas (mineralized nodule formation) were analyzed using ImageJ software (National Institutes of Health, USA). Experiments were carried out using six samples for each condition.

2.4. In vitro osteoclastogenesis

RAW264.7 cells were cultured in 96-well plates for 5 days in α-MEM medium containing 10% FBS and 100 ng/ml soluble receptor activator of NF-κB ligand (sRANKL, Peprotech). sRANKL (100 ng/ml) was also added to BMM (5×10^6 cells) cultured in 96-well plates for 10 days with α-MEM medium containing 10% FBS and 50 ng/ml M-CSF.

A standard tartrate-resistant acid phosphatase (TRAP; a marker enzyme of osteoclasts) staining [23] was performed to evaluate osteoclast formation. TRAP-positive dark-red cells with more than three nuclei were counted as multinucleated osteoclasts under light microscopy. Experiments were carried out using eight samples for RAW264.7 cells and six samples for BMM in each condition.

The amount and activity of TRAP form 5b (TRAP 5b) in the cell culture supernatants were also analyzed by an enzyme-linked immunosorbent assay (ELISA) using a mouse TRAP assay kit (SBA Sciences, Turku, Finland) [27]. Experiments were carried out using three samples for RAW264.7 cells and six samples for BMM in each condition.

Total RNA was extracted to compare the expression of osteoclastogenic marker genes (calcitonin receptor, cathepcin K, TRAP) based on reverse transcription–polymerase chain reaction (RT–PCR) analysis. For the NFAT reporter assay, the luciferase activity of each well was measured using the ONE-Glo™ luciferase assay system (Promega) and a microplate reader (GloMax-Multi Detection System, Promega). The reporter assay was carried out in quadruplicate for each condition.

2.5. RT–PCR analysis

Total RNA was extracted with an RNeasy Mini Kit (QIAGEN, Hilden, Germany). After DNase I treatment (Ambion, Austin, TX), cDNAs were synthesized from 2 μg of total RNA using Super ScriptIII reverse transcriptase (Invitogen, Carlsbad, CA). The cDNA target was amplified by PCR using *Taq* DNA polymerase (Promega) following the manufacturer's recommendations. PCR conditions and primer pairs used are given in Table 1. PCR products were subjected to a 1.5% agarose gel electrophoresis with ethidium bromide staining and visualized under ultraviolet light illumination (Dolphin-View Image System: Wealtec, Sparks, NV). Expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control.

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