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Isolation and characterization of a novel peptide, osteoblast activating peptide (OBAP), associated with osteoblast differentiation and bone formation

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ABSTRACT

A long-standing goal in bone loss treatment has been to develop bone-rebuilding anabolic agents that can potentially be used to treat bone-related disorders. To purify and isolate a novel anabolic that acts to osteoblasts, we monitored changes in intracellular calcium concentrations ($[Ca^{2+}]i$). We identified a novel, 24 amino-acid peptide from the rat stomach and termed this peptide osteoblast activating peptide (OBAP). Furthermore, we examined the effects of OBAP in osteoblasts. First, osteoblast differentiation markers (alkaline phosphatase [ALP], osteocalcin [OCN]) were analyzed using quantitative RT-PCR. We also examined the ALP activity in osteoblasts induced by OBAP. OBAP significantly increased the expression of osteoblast differentiation markers and the activity of ALP *in vitro*. Next, to address the *in vivo* effects of OBAP on bone metabolism, we examined the bone mineral density (BMD) of gastrectomized (Gx) rats and found that OBAP significantly increased BMD *in vivo*. Finally, to confirm the *in vivo* effects of OBAP on bone, we measured serum ALP and OCN in Gx rats and found that OBAP significantly increased serum ALP and OCN. Taken together, these results indicate that the novel peptide, OBAP, positively regulates bone formation by augmenting osteoblast differentiation. Furthermore, these results may provide a new therapeutic approach to anabolically treat bone-related disorders.

1. Introduction

Osteoporosis is characterized by low bone mass and structural bone deterioration, both of which are associated with reduced bone strength and an increased risk of fracture. In the United States alone, 10 million people are estimated to have osteoporosis, and osteoporosis contributes to 1.5 million fractures each year [1]. Bone undergoes a normal remodeling process, mediated by the coordinated actions of osteoclasts and osteoblasts. Bone loss and skeletal fragility in osteoporosis are caused by an imbalance in bone remodeling, in which the rate of osteoclast-mediated bone resorption is higher than the rate of osteoblast-mediated bone formation. A variety of therapeutics are currently used to treat osteoporosis, with the vast majority being antiresorptive agents that exert their clinical effects by decreasing the rate of bone resorption, thereby preventing further bone loss and reducing fractures [2]. Along with ongoing efforts to develop improved antiresorptive agents, a long-standing goal has been to develop therapeutics that can stimulate bone formation to increase bone mass and bone strength. It is thought that such bone-rebuilding anabolics could provide important treatment options, not only for low bone mass conditions but also for fracture healing, orthopaedic procedures, etc. Currently, the only approved bone anabolic agents for osteoporosis are full-length and truncated PTH, both of which are administered by daily subcutaneous injections [2]. Therefore, it is important to identify new bone anabolic agents.

Here, we report the purification and isolation of a novel peptide that acts to osteoblasts. In addition, we demonstrate that this novel peptide regulates osteoblast activation and bone formation.

2. Materials and methods

2.1. Animals

Sprague–Dawley (SD) rats (Charles River Co., Yokohama, Japan) were used to purify the peptide and to conduct both osteoblast-like cell culture experiments and *in vivo* studies. The rats were housed in a regulated environment (22 ± 2 °C, 55 ± 10% humidity, 12-h light, 12-h dark cycle with lights on at 0700 h) with free access to food and water. All experiments were conducted in accordance with the Japanese Physiological Society's guidelines for animal care.

2.2. Cell culture

Primary osteoblast-like cells were isolated by digesting a 21-day-old fetal rat calvaria with collagenase (Sigma Chemical

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Co., St. Louis, MO, USA) as previously described [3,4]. Digests 3–5 were pooled and grown in 10-cm cell culture plates in primary culture media consisting of α-Minimal Essential Medium (α-MEM; containing L-glutamine and nucleosides) (Life Technologies-GIBCO, Cergy Pontoise, France) supplemented with 10% fetal bovine serum (Thermo Trace, Melbourne, Australia) and antibiotics, including 100 μg/ml penicillin G (Life Technologies-GIBCO), 50 μg/ml streptomycin sulfate, and 0.3 μg/ml Fungizone (Life Technologies-GIBCO). Cells were grown to confluence before experimentation.

UMR-106 cells, a rat osteoblastic cell line, were obtained from Dainippon Pharmaceutical Co. (Osaka, Japan). Cells were plated in 10-cm plates at a density of 2×10^5 cells/plate and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies-GIBCO) supplemented with 10% fetal bovine serum and antibiotics.

All cultures were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.3. Purification of the novel peptide

The novel peptide was isolated and purified by the same method as previously described [5]. A fresh rat stomach (50 g) was diced and boiled for 5 min in five volumes of water to inactivate the intrinsic proteases. The solution was adjusted to 1 M AcOH, 20 mM HCl. Peptides were extracted by homogenizing with a Polytron mixer. The extracts were centrifuged for 30 min at 11,000 rpm, and the supernatants were concentrated to approximately 40 ml with an evaporator. The residual concentrate was precipitated with 66% acetone. After the precipitates were removed, the acetone-containing supernatant was evaporated. The solution was loaded onto a 10-g cartridge of Sep-Pak C18 (Waters), which was pre-equilibrated with 0.1% trifluoroacetic acid (TFA). The Sep-Pak cartridge was washed with 10% CH_3CN/0.1% TFA, and then eluted with 60% CH_3CN/0.1% TFA. The eluate was evaporated and lyophilized. The residual materials were redissolved in 1 M AcOH and then adsorbed on a SP-Sephadex C-25 column (II + form) that was pre-equilibrated with 1 M AcOH. Successive elution with 1 M AcOH, 2 M pyridine and 2 M pyridine AcOH (pH 5.0) yielded three fractions of SP-I, SP-II, and SP-III. The lyophilized SP-III fraction was applied on a Sephadex G-50 gel-filtration column. A portion of each fraction was subjected to the intracellular calcium concentration ($[Ca^{2+}]i$)-change assay using UMR-106 cells. Active fractions (43–48) were then separated by reverse-phase high-performance liquid chromatography (HPLC) using a symmetry300 C18 column (3.9 × 150 mm; Waters). The active fractions were further fractionated manually.

2.4. Structural analysis

The amino-acid sequence of the peptide was analysed with a protein sequencer (494; Applied Biosystems, Foster City, USA). The molecular weight was determined using MS. The fully protected peptide was synthesized by the Fmoc solid-phase method on a peptide synthesizer (433A; Applied Biosystems). Purified peptides were compared to synthetic peptides by reverse-phase HPLC. The activities of the novel peptide, HPLC-fractionated peptides, and the synthetic peptides were examined by monitoring the effects of these samples in the $[Ca^{2+}]i$-change assay.

2.5. Changes in intracellular Ca^{2+} concentrations

Changes in $[Ca^{2+}]i$ were measured using the FLEXstation Calcium Assay Kit (Molecular Devices), fluorescent dye Fluo-4 AM (Molecular Probes), and black-walled, clear-bottomed 96-well microplates (Costar, cat# 3603 and Greiner, E&K cat# 655090). Compound plates used were 96-well, V-bottomed, clear polypropylene plates (E&K, cat# 651201). A 1X Reagent Buffer containing 1× Hank's Balanced Salt Solution and 20 mM HEPES pH 7.4 was used to wash cells, dilute compound, and dissolve dye solutions. All buffers contained a final concentration of 2.5 mM probenecid in order to inhibit endogenous efflux pumps. Cells were seeded the night before the experiment at 5×10^4 cells/well in a volume of 100 μl per well of a 96-well microplate. Cells were incubated at 37 °C in 5% CO_2 overnight. The next day, the cells were incubated with Fluo-4 dye loading buffer at room temperature for 1 h. Then, the Fluo-4 dye-loaded cells were manually washed three times with 1× Reagent Buffer, and 100 μl of 1× Reagent Buffer was added to the wells. The compound was added to the plates of cells via FLEXstation and subsequent changes in fluorescent signals were monitored.

2.6. Cell proliferation assays

Primary osteoblast-like cells were seeded in 96-well plates at a density of 6000 cells/well. After 24 h, the media was changed to serum-free medium with 1% bovine serum albumin, and the cells were incubated for an additional 24 h before the experimental compounds were added.

The relative number of viable cells in each well was determined after a 48-h incubation with the compounds using the cell count reagent SF (Nacalai Tesque, Kyoto, Japan), 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8). Briefly, 10 μl of the WST-8 solution was added to each well, including three wells containing medium alone that were used to subtract the background fluorescence. Then, the cells were incubated at 37 °C for 1 h. The absorbance at 450 nm in each well was determined using a microplate reader, model 550 (Bio-Rad Laboratories, Hercules, CA, USA). This technique produces a linear relationship between the number of viable cells and the absorbance at 450 nm.

2.7. Assessment of the expression of osteoblast differentiation markers

Cells in 6-well plates were treated with or without peptide (10^{-4} or 10^{-5} M) and then analyzed after 3 and 14 days of *in vitro* culture. Cells were plated at a density of 4×10^4 cells/well and grown until they reached confluency, which was designated day 0. Cells were grown in primary culture media with 50 μg/ml ascorbic acid (Sigma Chemical Co.) and 10 mmol/L β-glycerophosphate (Sigma Chemical Co.). Total RNA was extracted from the cell pellets using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA using the Super Script Preamplification System for First-Strand cDNA Synthesis Kit (Life Technologies-GIBCO, Cergy Pontoise, France). cDNA was analyzed by quantitative real-time PCR using the ABI PRISM 7700 sequence detection system (Applied Biosystems). Specific primers that produce short PCR products suitable for SYBR-Green detection were designed using Primer Express software (version 1.0, PE Applied Biosystems). The primer sequences were as follows: Runx2 (Runx2; 67-bp product; GenBank accession no. AF053953), 5′-GCTTCATTCGCCTCACAAACA-3′ (sense) and 5′-TGCTGTCCTCCTGGAGAAAGTT-3′ (antisense); alkaline phosphatase (ALP; 101-bp product; GenBank accession no. J03572), 5′-CGTCTCCATGGTGGATTATGC-3′ (sense) and 5′-TGGCAAAGACCGCCACAT (antisense); osteocalcin (OCN; 63-bp product; GenBank accession no. X04141), 5′-GAGCTAGCGGACCACATTGG-3′ (sense) and 5′-CCTAAACGGTGGTGCCATAGA-3′ (antisense); and beta-actin (β-actin; 67-bp product; GenBank accession no. NM031144), 5′-TTCAACACCCCAGCCATGT-3′ (sense) and 5′-GTGGTACGACCAGAGGCATACA-3′ (antisense). Samples were examined in triplicate. The reaction volume was 50 μl and included 3 μl diluted cDNA (1:30), 10 μl SYBR green buffer, and 10 pmol of each primer. The samples were subjected to 45 cycles of amplification at 95 °C for 15 s, followed