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Original Article

The Effect of α MSH Analogues on Rat Bones

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Melanocortin is the downstream mediator of leptin signaling and absence of leptin signaling in ob/ob and db/db mice revealed the enhancement of bone formation through the central regulation. While alpha-melanocyte-stimulating hormone (α MSH) inhibits the secretion of interleukin-1 α and tumor necrosis factor-α from the inflammatory cells, α MSH can also enhance clonal expansion of pro B cells linked to stimulation of osteoclastogenesis. Therefore, we tested the effect of melanocortin on bones. α MSH analogues [^{6}His] α MSH-ND and [^{6}Asn] α MSH-ND were synthesized and the radio-ligand receptor binding- and cyclic AMP generating activity were analyzed in China Hamster Ovary cell line over- expressing melanocortin receptors. The EC_{50} of [^{6}His] α MSH-ND measured from melanocortin-1, 3, 4 and 5 receptors were 0.008 ± 0.0045, 1.523 ± 0.707, 0.780 ± 0.405, and 250.320 ± 42.234 nM, respectively, and the EC_{50} of [^{6}Asn] α MSH-ND were 16.8 ± 6.94, 271.8 ± 21.95, 8.0 ± 1.21, and 1132.5 ± 635.46 nM, respectively. Four weeks after the subcutaneous injection of the analogues, the body weights in the [^{6}His] α MSH-ND and the [^{6}Asn] α MSH-ND treated groups (346.0 ± 20.63 g vs. 350.0 ± 13.57 g) were lower than that of the vehicle treated group (375.8 ± 17.31 g, $p < 0.05$). There was no difference in the total femoral BMD measured by dual x-ray absorptiometry among the three groups. Among the three groups, there were no differences in the total numbers of crystal violet positive- or alkaline phosphatase positive colonies, in the expression of Receptor Activator of Nuclear Factor Kappa-B ligand on the tibia and the total number of multinucleated osteoclast-like cells differentiated from primary cultured bone marrow cells. From the above results, no evidence of bone gain or loss was found after treatment of the α MSH analogues peripherally.

Key Words: α MSH, bone mineral density, osteoclast

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INTRODUCTION

In the absence of a functional melanocortin-4 receptor (MC4R), animals cannot respond to increasing leptin concentrations in the blood, which results in the development of severe obesity.[1] Alpha-melanocyte-stimulating hormone (α MSH) derived from the pro-opiomelanocortin neurons in the arcuate nucleus, which is the primary source of MC4R ligands, appears to play an inhibitory role in feeding and energy expenditure while neuropeptide Y (NPY) and the Agouti related protein are important mediators in the response to starvation.[2,3] Recently, the absence of leptin signaling in ob/ob and db/db mice revealed enhanced bone formation through central regulation.[4,5] To clarify the precise nature of the central inhibition of bone formation by leptin, the ob/ob mice were treated with an intracerebroventricular NPY infusion.[5] Unexpectedly, NPY induced bone loss instead of bone gain, indicating that leptin and NPY do not antagonize each other in controlling bone formation. This suggests that leptin may use different sets of mediators to control body weight and bone mass.[5] However, the potential role of melanocortin on the bone was not investigated.

Regarding osteoclastogenesis, the effects of α MSH on melanocortin-1 receptor (MC1R) and melanocortin-5 receptor (MC5R) might be opposite. MC1R activation by α MSH induced anti-inflammatory effects by inhibiting the formation of inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and stimulating the formation of anti-inflammatory cytokines such as interleukin-10 (IL-10), interleukin-8 (IL-8).[6] The increase in

osteoclastogenesis induced by an estrogen deficiency was related to the increased production of multiple cytokines including IL-1, IL-6 and TNF-α.[7] MC1R activation can also inhibit inducible nitric oxide (NO) synthase and the activation of nuclear factor kappa B (NF-*k*B) brought about by a variety of inflammatory stimuli, namely TNF-α, bacterial lipopolysaccharide, okadeic acid and ceramide.[8] Recently, a non-receptor mediated mechanism of α MSH on inflammatory processes was also proposed because the tripeptide fragment of a MSH (11-13) analogue, which is not known to bind to MC1R, was able to mimic many effects caused by α MSH on inflammatory processes such as inhibiting the binding of radiolabelled IL-β to IL-1 receptor.[9]

The role of MC5R in activating the tyrosine phosphorylation pathway, Janus kinase-signal transducer and activator of transcription (JAK/STAT), was proposed for pro B cells, which only express MC5R.[10] α MSH was shown to promote tyrosine phosphorylation of JAK2, a member of the intracellular JAK tyrosine kinase family and to induce proliferation of B-lymphocytes.[10] There is evidence that the clonal expansion of the B-cell precursor was followed by increased proliferation of osteoclast precursor cells and bone loss in mice.[11]

Melanocortin is the down stream signal of leptin and the precise nature of the central inhibition of bone formation by leptin was not defined. Furthermore, melanocortin is one of the target molecule for treatment of obesity, therefore, it should be addressed whether treatment of melanocortin peripherally could affect bone formation or not. One might speculate that the effects of α MSH on MC1R and MC5R regarding osteoclastogenesis are opposite from the effects of melanocortin on inflammation, however, there are few data showing the direct effects of melanocortin on osteoclastogenesis. Therefore, we tested *in vivo* effects of the α MSH analogues on the bone by analyzing the bone mineral density (BMD) and the histologic findings in the femurs. In addition, their effects on monocyte colony stimulating factor (M-CSF) & RANKL mediated osteoclastogenesis and on differentiation of osteoblast from cultured bone marrow cells were also studied.

MATERIALS AND METHODS

Animals and treatment

Twenty one male Sprague-Dawley rats, approximately 8 weeks of age and weighing approximately 180-200 gm, were used. The rats were randomly divided into three groups. Each rat was provided food and water ad libitium. The vehicle in group 1, a dose of 100 nM of [^{6}His] α MSH-ND in group 2 or [^{6}Asn] α MSH-ND in group 3 were injected subcutaneously twice a day for 4 weeks. The rats were sacrificed four weeks after treatment. The entire femoral bones were removed and the right femur was used for the BMD measurements by using a dual x-ray absorptiometry (DXA) (Hologic QDR 4500A, Waltham, MA, USA). The left femur was used for light microscopic examination and the left tibia was used for immunohistochemistry. The left femoral bone was fixed in a 5% formalin solution.

The vehicle or a dose of 100 nM of [^{6}His] α MSH-ND or [^{6}Asn] α MSH-ND was injected intraperitonealy prior to treatment and sacrifice to examine the long-term effect of the melanocortin analogues on the daily food intake.

All animals were treated in accordance with the guidelines and regulations for the use and care of animals of Yonsei University, Seoul, Korea.

Chemicals and peptide synthesis

The peptides used in this study were synthesized at the Korea Basic Science Institute (Seoul, Korea) using the solid phase approach and purified by high performance liquid chromatography (HPLC) (Delta PAK 15 μ, C18 300Å, 3.9 × 150 mm column, detection at 240 nm). The peptide sequences were assembled with a Milligen 9050 (Fmoc Chemistry). Mass spectroscopy (MS) was used to determine the molecular masses of the peptide. For deprotection, a reagent mixture (88% trifuloroacetic acid, 5% phenol, 2% triisopropylsilane, 5% H_2O; 2h) was used. The raw peptides were purified by HPLC. All media and sera for cultivation were purchased from Gibco-RL. The antibodies against RANKL (RANKL FL-317) were purchased from Santa Cruz Biotechnology, Inc.

Expression of melanocortin receptor clones and cell culture

Both human MC1R (hMC1R) and human MC5R (hMC5R) cDNA were cloned into the expression vector, pCDNA I neo. To investigate receptor expression, the CHO cells were grown in a F-12 medium with 10% fetal bovine serum (FBS) and 95% O_2 air/5% CO_2 and transfected with hMC1R and hMC5R -pCDNA neo, respectively, by the calcium phosphate method. Briefly, 5-7 $\times 10^5$ cells (approximately 80% confluent cultures) were plated in 10-cm culture dish the day before they were transfected. The cells were fed with fresh complete culture medium containing 20 nM Hepes and incubated at 95% O_2 air/5% CO_2. After 3 - 4 h, the medium was discarded and 5 ml of a calcium phosphate-DNA precipitate containing 25 μg DNA, 124 mM $CaCl_2$, 140 mM NaCl, 25 mM Hepes, and 1.41 nM Na_2HPO_4 (pH 7.12) was added. The cells were incubated for 4 h with 97% O_2 air/3% CO_2, and washed with NaCl/Pi (137 mM $NaCl_2$, 2.68 mM KCl, 4.3 mM $NaHPO_4$, 1.47 mM KH_2PO_4, pH 7.12). They were then shocked with a glycerol buffer (15% glycerol, 140 mM NaCl, 25 mM Hepes and 1.41 mM Na_2HPO_4, pH 7.12). They were again washed with NaCl/Pi and incubated for an additional 36 - 48 h in complete F-12 medium. The cells were then cultured in complete F-12 medium containing 0.5 mg/ml G418 (Geneticin; Life Technology) until the G418 resistant colonies were selected and subcultured for at least 10 - 14 days. The hMC1R and hMC5R- expressing cells were identified by screening more than 15 colonies and was confirmed by an assay of [Ahx^4] α MSH-induced cyclic AMP accumulation. The rat MC3R (rMC3R), hMC4R and hMC5R-expressing cells were established using the same method used previously.[12]

Binding study

Iodinated NDP-MSH (the NDP-MSH sequence is Ser-Tyr-Ser-Ahx-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val), ^{125}I(Iodotyrosyl^2)-[Ahx^4, D-Phe^7] α-MSH, was prepared by the modified chloramine-T method, as described previously.[13] 1mCi (10 μl) of $Na^{125}I$ (Amersham) was added to 5 μg of NDP-MSH in 100 μl of 200 mM sodium phosphate buffer (pH 7.2). 20 μl of 2.8 mg/ml chloramine T solution in 200 mM sodium phosphate (pH 7.2) was then added and the mixture allowed to stand for 15 seconds, followed by the addition of 50 μl of 3.6 mg/ml of sodium metabisulfate to stop the reaction. The reaction mixture was diluted with 1ml of 0.1% BSA solution containing 0.1% of trifluoroacetic acid and purified using a C18 Sep-Park cartridge (Waters) and Sephadex G25 Gel Filtration Chromatography. The purified reagents were collected in 0.5 ml aliquots in Sigmacote-coated sterile glass tubes containing 100 μl of PBS buffer with 0.1% bovine serum albumin. For binding assays, the stably transfected CHO cells were plated 48 hours before experimentation in 24-well culture plates (Falcon Plastics) at a density of 5 $\times 10^4$ per well until they were 90 - 95% confluent on the day of the assay. Maintenance media was removed and the cells were washed twice with washing buffer (50 mM Tris, 100 mM NaCl, 5 mM KCl, and 2 mM $CaCl_2$, at pH 7.2), and then immediately incubated at 37℃ for 2 hours with 0.25 ml binding buffer per well (50 mM Tris, 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5% Hanks' Balanced Salt Solution, and 0.5% Bovine Serum Albumin, at pH 7.2) containing 100,000 CPM (~2nM) of [^{125}I]NDP-MSH and appropriate concentrations of the unlabeled competing ligand. After incubation, the plates were placed on ice for 15 min, washed twice with 0.5 ml of ice-cold binding buffer, and detached from the plates by twice flushing with 0.5 ml of 0.05 M NaOH (final volume: 1 ml). Radioactivity was then determined (Workman automatic gamma counter) and data analyzed with a software package suitable for radioligand binding data analysis (GraphPad Prism Program). Nonspecific binding was determined by measuring the amount of bound [^{125}I] NDP-MSH remaining in the presence of 10^{-5}M unlabeled NDP-MSH, while specific binding was calculated by subtracting the nonspecifically bound radioactivity from total bound radioactivity. *Ki* (nM) values were calculated using the Cheng & Prusoff equation,[14] and represented as mean ± S.E. All of the binding assays were performed in triplicate wells and repeated twice.

Cyclic AMP stimulation in CHO cells expressing melanocortin receptors

The China hamster ovary (CHO) cells expressing the receptors were grown to confluence in 24-well plates. The cell culture medium was changed to complete F-12 medium containing 10% FBS 3-4 h before the cells were treated with the peptides. For the assays, the medium was removed and the cells were washed with 0.5 ml cAMP generating media containing 10% FBS, 2 mM 3-isobutyl-1-methylxanthine, 0.1% BSA, 20 mM Hepes, 0.002% ascorbic acid in complete F-12 medium. The cAMP generating media (0.25 ml) containing various concentrations of peptides were added and the cells were incubated for 30 minutes at 37℃. The media were then discarded, and the cells were frozen at -70℃ for 30 minutes and thawed at room temperature for 15 - 20 min. The process of freezing and thawing was repeated twice. Subsequently, the cells were detached from the plate with 1 ml of a 50 mM HCL solution per well, transferred to a 1.5 ml Eppendorf tube, and centrifuged for 10 minutes centrifugation at 1900 × g. The supernatant was diluted 50 fold with the radioimmunoassay (RIA) buffer and the cAMP concentration was measured using a cAMP ^{125}I RIA kit (INCSTAR) according to the manufacture's instructions. The data was fitted to a sigmoid curve with a variable slope factor using nonlinear square regression in a GraphPad Prism. The EC_{50} values are expressed as mean ± SE. All the cAMP assays were performed in triplicate wells and repeated twice.

Bone mineral density

The BMD of the excised femurs was measured by dual energy x-ray absorptiometry (QDR-4500A, Hologic, Waltham, MA, USA). Triplicate determinations of the five different femurs with repositioning, showed a coefficient of the variation at 0.59%.

The first three hour-cumulative food intake

The rats were maintained on a normal 12 hr/12 hr light/dark cycle with food and water ad *libitium*. The animals were housed individually in metabolic cages for at least 24 hr prior to the injection. They were then fasted during the night (6 pm to 8 am) to stimulate feeding during the daytime experimental period. The rats were distributed into weight-matched control and experimental groups with 7 rats per group, and injected intraperitoneally with the vehicle (saline, 100 μl each rat) or the vehicle plus the drug ([^{6}His] α MSH-D/[^{6}Asn] α MSH-ND, 100 nM/100 μl saline) as indicated. A twelve-hour feeding assay was performed. Briefly, the quantity of food pellets in a spill-free cup was pre-measured. The food remaining was removed and weighed at timed intervals.

Immunohistochemistry

The excised tibia was decalcified for 48 - 72 h in several changes of acid/citrate buffer (13% sodium citrate in 2% formaldehyde, pH 4.7 with formic acid). The tissues were dehydrated then embedded in paraffin. Sequential sections near the midsagittal plane were cut 5 μm wide and air-dried on gelatin-coated slides. After deparaffinization and antigen unmasking, RANKL Ag immunoperoxidase staining was performed following the recommended protocol of the Santa Cruz Biotechnology ABC staining system. The sections were mounted in Aquamount (BDH Chemicals Ltd., Poole, UK) for light microscopy.

Osteoblast cell culture

Bone marrow stromal cells were cultured as described previously.[15] The rats were sacrificed by a cervical dislocation, and the tibiae were aseptically removed and dissected free of adhering tissues. The bone ends were removed with scissors and the cavity was flushed with 1 ml α-MEM by a slow injection from one end of the bone using a sterile 26 G needle. The resulting medium with flushed bone marrow was then centrifuged at 1400 rpm for 10 minutes. The cell pellets were resuspended in culture medium and layered on Ficoll/Hypaque (specific gravity 1.077). After centrifuging at 1400 rpm for 30 minutes, an enriched bone marrow stromal cell fraction was obtained from the interface of the culture medium and the Ficoll / Hypaque layer. The cells were seeded onto a 35 mm tissue culture dish at a den-

sity of 4×10^5 cells/cm^2 and cultured in α- MEM containing 10% FBS, penicillin, and streptomycin (100 U/ml and 100 μg/ml, respectively). The medium was changed twice weekly from the second week. The cells were subcultured using conventional techniques employing 0.01% trypsin and 0.05% EDTA when they grew up to 80% confluence.

In vitro osteoblast cell differentiation assay

Primary cultured marrow cells were split into 2×10^6 / T25 Flask and cultured for 2 or 3 weeks in α-MEM with 10% FBS, 10^{-8} M dexamethasone, 50 μg/ml ascorbate-2-phosphate, and 2 mM β-glycerolphosphate. Depending on the particular experiment, either 10^{-6} M [^{6}His] αMSH-ND or [^{6}Asn] αMSH-ND was applied to each well. Two weeks after treatment, the cells were fixed with formalin at 4℃. After washing three times with PBS, the cells were stained with a crystal violet working solution {1/10 of [crystal violet (90-95%), ethanol and ammonium oxalate (1%)]} for 5 minutes to identify the CFU-F positive colonies. Colonies having more than 50 cells/colony were counted. The alkaline phosphatase(ALP)-positive colonies were also counted after ALP staining. The ALP staining method followed the protocol provided by Sigma (ALP Kit, Sigma 85L-1).

In vitro osteoclastogenesis

The preosteoclasts (pOCs) were prepared using an osteoclastogenesis as previously described.[12] Briefly, ICR mouse bone marrow cells (2×10^7 cells) were cultured in α-MEM containing 10% fetal calf serum in a 10-cm dish for 1 day. The mononuclear cells attached to dish were retrieved by gentle pipetting with fresh α-MEM. These cells were collected by centrifugation (1000 rpm, 5 minutes) and were used for preparing the pOCs. The pOCs population constituted more than 50% of the total cells in the tartrate resistant acid phosphatase (TRAP) stain. However, there were no multinucleated cells having more than three nuclei. In addition, no osteoblastic cells were detected by staining for alkaline phosphatase. The pOCs (1×10^5 cells/well) were cultured in the presence or absence of 10^{-6} M [^{6}His] αMSH-ND or [^{6}Asn] αMSH-ND with M-CSF and RANKL (each at 100 ng/ml) in 48 well cell culture plates. After culturing for 72 h, the cells were fixed and stained for TRAP. TRAP-positive cells with more than four nuclei were counted.

Statistical analysis

SAS software (6.12, for windows, North Carolina) was used. The data was expressed as the means ± S.E. A Student's t-test was used to determine the statistical significance of changes in the binding affinity and peptide potency. ANOVA was used for analysis of other values including body weight, food intake, BMD and numbers of cells. p value less than 0.05 was considered significant.

RESULTS

Receptor-binding activity of [^{6}His] αMSH-ND and [^{6}Asn] αMSH-ND

The [^{6}His] αMSH-ND and [^{6}Asn] αMSH-ND binding affinities for MC1R, MC3R, MC4R and MC5R were measured. The [^{6}His] αMSH-ND Ki values (nM) on the MC1R, MC3R, MC4R and MC5R were 0.03 ± 0.020, 7.80 ± 1.501, 4.41 ± 0.704 and 450.32 ± 68.351, respectively (Fig. 1). The [^{6}Asn] αMSH- ND Ki values (nM) on the MC1R, MC3R, MC4R and MC5R were 151.38 ± 20.780, 330.22 ± 60.050, 112.57 ± 9.738, and 4436.36 ± 872.532, respectively.

Effects of [^{6}His] αMSH-ND and [^{6}Asn] αMSH-ND on cyclic AMP accumulation in CHO cells over-expressing MC1R, MC3R, MC4R or MC5R

The EC_{50} (nM) of [^{6}His] αMSH-ND to the MC1R, MC3R, MC4R and MC5R stably transfected with the CHO cell line were 0.008 ± 0.0045, 1.523 ± 0.707, 0.780 ± 0.405, and 250.32 ± 42.234 nM, respectively (Fig. 2). EC_{50} of [^{6}Asn] αMSH- ND to the MC1R, MC3R, MC4R and MC5R stably transfected with the CHO cell line were 16.8 ± 6.94, 271.8 ± 21.95, 8.0 ± 1.21, and 1132.5 ± 635.46 nM, respectively. Compared to [^{6}Asn] αMSH-ND, the potency of [^{6}His] α-MSH-ND was stronger in MC1R, MC3R and MC4R but was weaker in MC5R. [^{6}Asn] αMSH-ND was not capable of

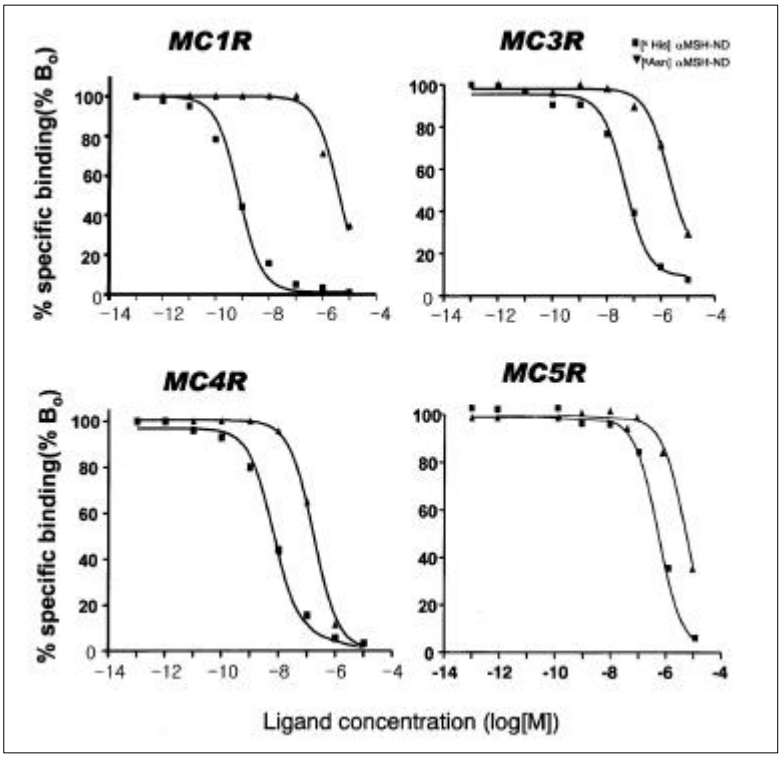

Fig. 1. Competitive radioligand binding assay of [^{6}His] α MSH- ND (■) and [^{6}Asn] α MSH-ND (▲) on MC1R, MC3R, MC4R and MC5R.

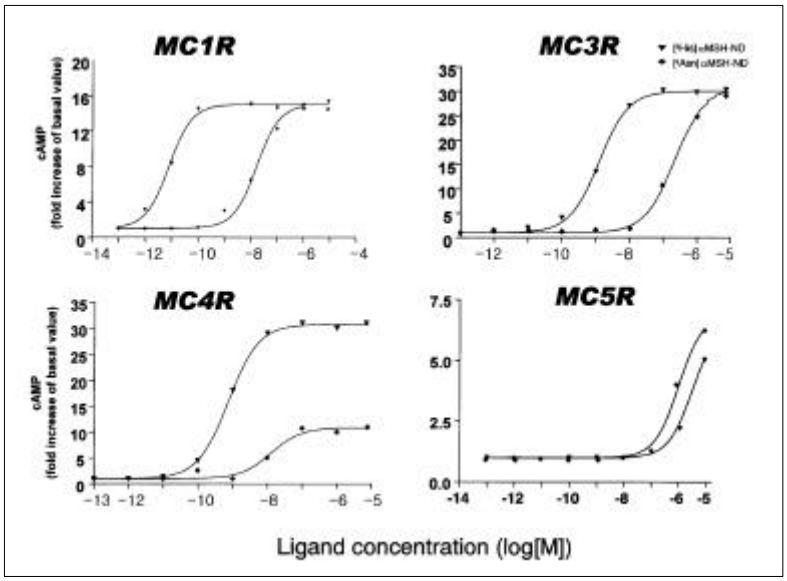

Fig. 2. Measurement of intra-cytoplasmic cAMP in response to increasing concentration of [^{6}His] α MSH-ND (▼) and [^{6}Asn] α MSH-ND (◆) on MC1R, MC3R, MC4R and MC5R.

generating the maximum cAMP response at MC4R.

Food intake and body weight

The rats were encouraged to feed by fasting them for 16 h prior to the intraperitoneal administration of [^{6}His] α MSH-ND. In comparison to the vehicle-injected animals, both the [^{6}His] α MSH-ND and [^{6}Asn] α MSH-ND injected animals consumed less food within one hour of administration (Fig. 3). Food intake was inhibited up to 3 h after administration ($p < 0.05$, data not shown), with cumulative food intake resuming 6 hr after treatment. The first three hour-cumula-

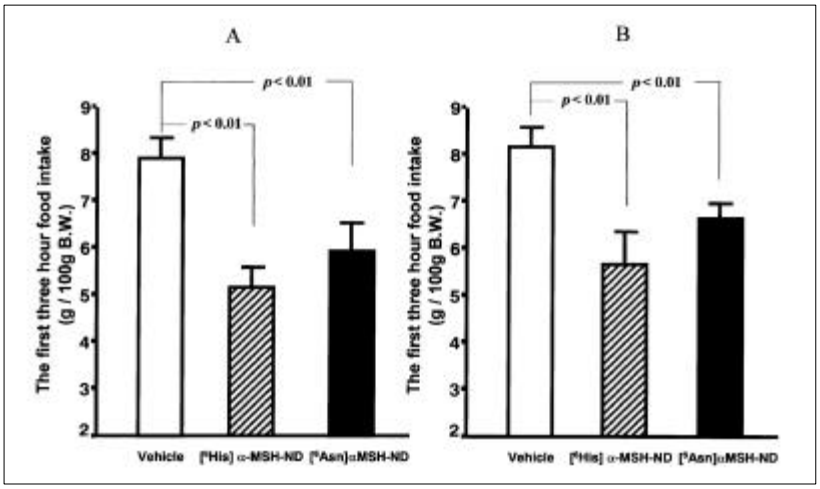

Fig. 3. The first three hour-cumulative food intake after single IP injection of vehicle, [^{6}His] α MSH- ND and [^{6}Asn] α MSH-D respectively before daily treatment (A) and 4 weeks after treatment (B).

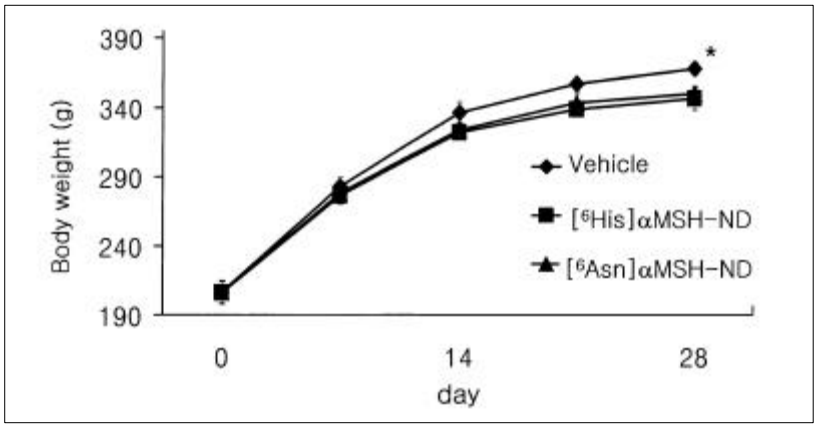

Fig. 4. Changes of body weight after subcutaneous injection of vehicle, [^{6}His] α MSH-ND and [^{6}Asn] α MSH- ND for 4 weeks ($*p < 0.05$).

tive food intake before the daily injection of the above test drugs were 7.9 ± 0.41 g, 5.2 ± 0.43 g and 5.9 ± 0.49 g / 100 g B.W., respectively. After 4 weeks of treatment, the first three hour-cumulative food intake before the daily injection of the test drugs were 8.2 ± 0.31 g, 5.7 ± 0.63 g and 6.6 ± 0.21 g/ 100 g body weight, respectively. The body weight of each group were 375.8 ± 17.31 g, 346.0 ± 20.63 g and 350.0 ± 13.57 g, respectively after a 4 week- treatment with the vehicle, [^{6}His] α MSH-ND or [^{6}Asn] α MSH-ND (Fig. 4). Compared to the vehicle treated group, the body weight was lower in the melanocortin treated groups ($p < 0.05$), but there was no difference in body weight between the [^{6}His] α MSH-ND and [^{6}Asn] α MSH-ND treated groups.

Bone mineral density difference

The weight matched total femoral BMD of each group was 0.231 ± 0.0035 g/cm^2, 0.231 ± 0.0058 g/cm^2, and 0.227 ± 0.0026 g/cm^2, respectively (Fig. 5). There was no significant difference among the three groups.

Histologic findings

On light microscopic examination, there were no significant differences in the microarchitecture of the cortical and trabecular bone, and in the number of mononuclear cells and multinucleated cells among the three groups. The RANKL was expressed in a scattered pattern at some bone marrow cells and chondrocytes of the epiphyseal growth plate, and there was no significant differ-ence in the rate of RANKL expression between the [^{6}His] α MSH-ND and [^{6}Asn] α MSH-ND treated group (Fig. 6).

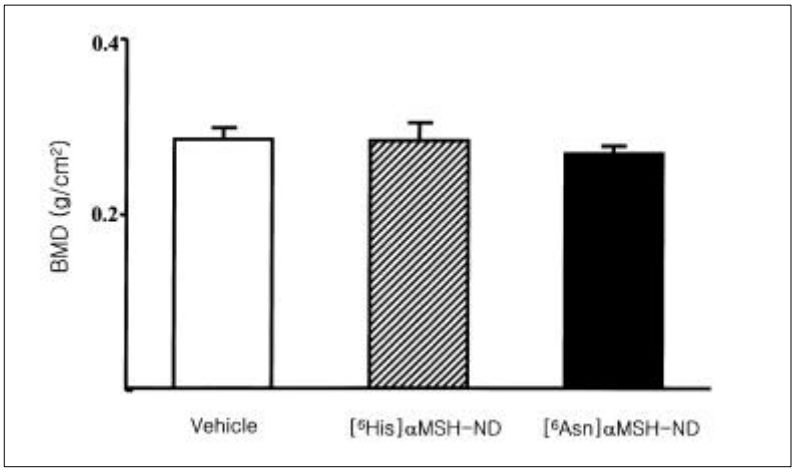

Fig. 5. Femoral BMD after treatment of vehicle, [^{6}His] α MSH-ND and [^{6}Asn] α MSH-D for 4 weeks.

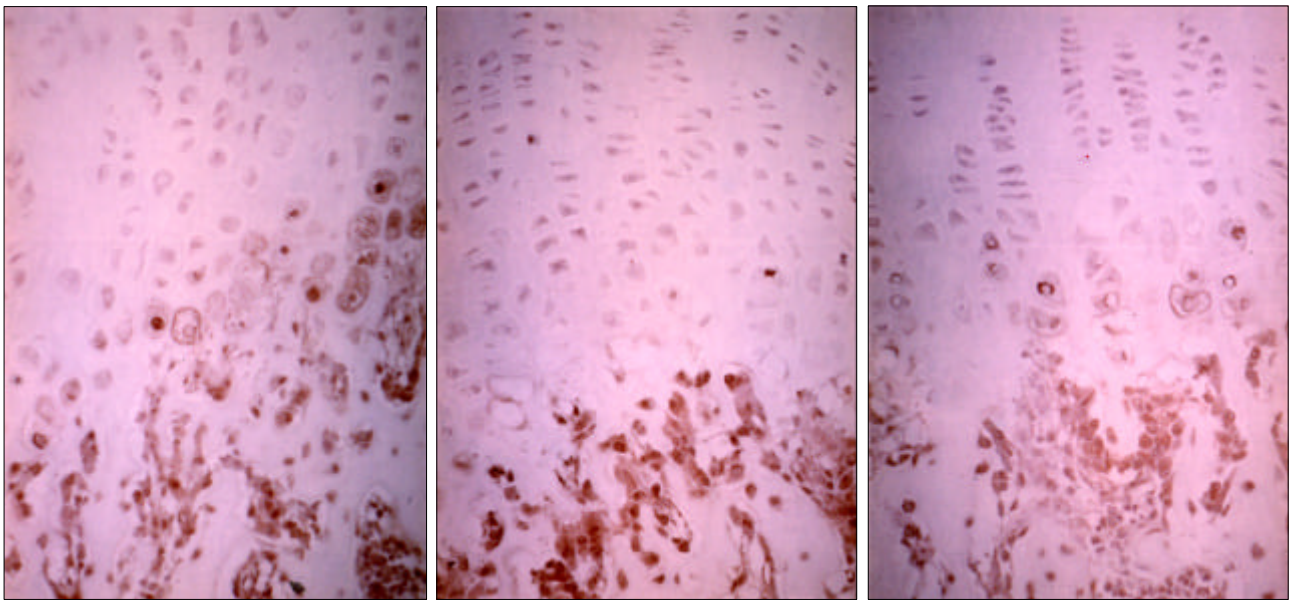

Fig. 6. Immunohistochemical analysis by using RANKL antibody in trabecular bone after treatment of vehicle(A), [^{6}His] α MSH-ND(B) and [^{6}Asn] α MSH-ND(C) [× 100]. (RANKL positive cells are expressed in red-brownish colors).

In vitro osteoblastogenesis

There were no significant difference in the number of CFU-Fs colonies, the mesenchymal cell indicator, and the number of ALP-positive colonies, the pre-osteoblast segmentation indicator, with [^{6}His] α MSH-ND and [^{6}Asn] α MSH-ND treatment (Table 1).

In vitro osteoclastogenesis

The primary bone marrow cells (ICR mice, 5-8 weeks of age) were cultured by the standard method with RANKL and M-CSF (Fig. 7). The vehicle or each of the α MSH analogues (10^{-6} M) was added to each well every 48 hours. There were no differences in the number of osteoclasts among the three groups (Vehicle: 276 ± 5.9/48 wells, [^{6}His] α MSH-ND: 280 ± 5.5/48 wells and [^{6}Asn] α MSH-ND: 282 ± 13.4/48 wells).

DISCUSSION

Melanocortins have been implicated in a multitude of physiological functions including ther

Table 1. The Effects of [^{6}His] αMSH-ND and [^{6}Asn] αMSH-ND on Differentiation of Osteoblasts

	Colony No.	
	Crystal Violet (+)	ALP (+)
Vehicle	68.5 ± 8.71	70.2 ± 1.72
[^{6}His] αMSH-ND	70.7 ± 1.37	70.5 ± 4.55
[^{6}Asn] αMSH-ND	69.2 ± 1.47	73.0 ± 5.93

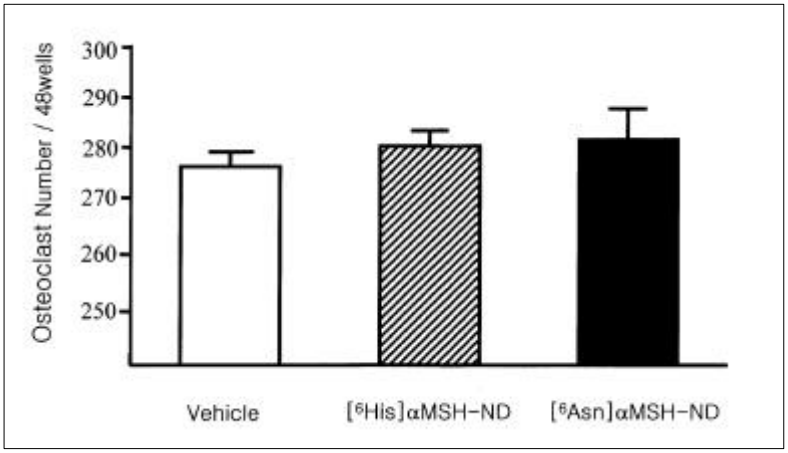

Fig. 7. The effect of [^{6}His] αMSH- ND and [^{6}Asn] αMSH-ND on the osteoclastogenesis induced by M-CSF and RANKL.

moregulation, obesity, control of the cardiovascular system, higher cortical functions related to attention, learning, and memory, and immunomodulatory effects. In the central regulation of fuel homeostasis, melanocortin is a down stream mediator of leptin signaling. Recently, Ducy et al. reported that leptin is a potent inhibitor of bone formation acting through the central nervous system. They proposed that there is a central nature of bone mass control and its disorder.[4] MC1R activation by αMSH induces an anti-inflammatory effect by inhibiting the formation of cytokines such as IL-1, IL-6 and TNF-α.[7] In contrast, clonal expansion of the B-cell precursor by activating MC5R in pro B cells has the potential to activate osteoclastogenesis.[10] Therefore, the effects of melanocortin on the bones should be clarified.

The core structure of αMSH is critically important for ligand-receptor interactions.[13,16] While αMSH revealed a hairpin loop conformation, the linear MTII ([^{6}His] αMSH-ND) prefers a type 1β turn conformation. Substituting ^{7}Phe of αMSH with ^{7}D-Phe plays a crucial role in making the type 1β turn conformation.[17] Because the substitution of ^{6}His by ^{6}Asn remarkably reduced the c-AMP generation activity in MC1R, two analogues, [^{6}His] αMSH-ND & [^{6}Asn] αMSH-ND were selected, and the radioligand-receptor binding- and c-AMP generating activities were compared in the CHO cell lines stably transfected with hMC1R, rMC3R, hMC4R or hMC5R. Compared to the binding- and c-AMP generation activity of both analogues at the MC1R expressing CHO cell lines, their activity at the MC5R expressing CHO cell lines were reduced remarkably. Furthermore, substituting ^{6}His of [^{6}His] αMSH-ND with ^{6}Asn did not show any significant difference in activity at the MC5R expressing CHO cell lines.

Compared to the control group, an intraperitoneal injection of [^{6}His] αMSH-ND reduced food intake during the first 3 hours, which continued for up to 6 hours after the injection (data not shown). A subcutaneous injection of 100 nM [^{6}His] αMSH-ND or [^{6}Asn] αMSH-ND twice daily for four weeks reduced the body weight. However, there was no difference between the two groups. The EC_{50} of [^{6}Asn] αMSH-ND was ~10 fold lower than that of [^{6}His] αMSH-ND in the CHO cell lines stably transfected with hMC4R, but it could

not stimulate c-AMP generation maximally. Although the reason for this is unclear, one of the plausible explanations might be the difference between the *in vitro* and *in vivo* effects of the α MSH analogues. The potency of the target gene-activation by the α MSH analogues might be different from the potency measured by the cAMP generating activity in CHO cell lines over expressing MC4R with phosphatase inhibitor (IBMX) treatment. Recently, it was reported that the luciferase activities measured after treating the CHO cells cotransfected with the MC4R and CRE-luciferase reporter system with [^{6}His] α MSH-ND or [^{6}Asn] α MSH-ND, were not different.[18]

Melanocortin treatment for 4 weeks did not make any difference in the weight matched BMD at all. Regarding the peptide dosage, an injection of 100 nM of α MSH twice daily might not be enough to inhibit food intake properly although it was enough to allow the anti-inflammatory effect of α MSH to be observed.[19] Ducy et al. reported no difference in bone mass in Agouti yellow (A^y/a) mice expressing the agouti gene obliquely compared to the control mice.[4] Agouti protein inhibits α MSH binding to MC1R and MC4R competitively. Therefore, their results implied that inhibiting α MSH did not affect the bone mass at all. Overall, this suggests that melanocortin is not involved in the central regulation of leptin-mediated inhibition of bone formation.

[^{6}His] α MSH-ND or [^{6}Asn] α MSH-ND did not affect the early differentiation of osteoblasts because the total number of crystal violet positive- or ALP positive colonies were not different among the three groups. Light microscopic examination of the bone marrow cells showed no difference in the number of multinucleated cells among the three groups. Even though RT-PCR or Northern blot analysis might be a better choice, we addressed the expression of RANKL by immunohistochemistry. The RANKL was expressed in a scattering pattern in some of the bone marrow cells and chondrocytes of epiphyseal growth plate, and there was no difference at the level of RANKL expression between the [^{6}His] α MSH-ND or [^{6}Asn] α MSH-ND treated group.

The RANKL expressed in the stromal cells and osteoblasts is a member of the membrane associated tumor necrosis factor ligand family and induces osteoclast formation from the osteoclast progenitors.[20-22] Co-treatment of soluble RANKL and M-CSF could induce osteoclast cell differentiation from progenitor cells without direct contact of the stromal cells or osteoblasts.[23] MC1R activation can inhibit the activation of NF-*k*B brought about by a variety of inflammatory stimuli, namely TNF-α, bacterial lipopolysaccharide, okadeic acid and ceramide.[24] Osteopetrosis was developed by NF-*k*B1 and NF-*k*B2 double knock out mice, and it was suggested that NF-*k*B activation was important for osteoclastogenesis.[24] Recently, MC1R expression was reported in *RAW-264* monocyte cell lines, which can be made to transform to osteoclasts.[25] However, osteteoclastogenesis induced by soluble RANKL and M-CSF was not inhibited by [^{6}His] α MSH-ND or [^{6}Asn] α MSH-ND (10^{-6} M). The EC_{50} of [^{6}Asn] α MSH-ND was ~2,100 fold lower than that of [^{6}His] α MSH-ND in the CHO cell lines stably transfected with hMC1R. One explanation might be that MC1R was either not expressed at all in cultured bone marrow cells or the level of expression was extremely low. Whatever the mechanisms, our *in vivo* and *in vitro* results suggested that osteoclastogenesis is not affected by treatment with the melanocortin analogues with potent activity on MC1R.

A role of MC5R in activating the tyrosine phosphorylation pathway, JAK/STAT was proposed in proB cells, where only MC5R was expressed.[10] α MSH was shown to promote the tyrosine phosphorylation of JAK2, a member of the intracellular JAK tyrosine kinase family and it induced B-lymphocyte proliferation.[11] Increased proliferation of osteoclast precursor cells and clonal expansion of the B-cell precursor followed by bone loss was observed after estrogen deficiency, which could be mimicked by the repletion of IL-7 in the cultured B cell precursor.[11] However, lymphocytosis and osteoclastogenesis was not observed in cultured bone marrow cells after treatment with the α MSH analogues. The EC_{50} of [^{6}Asn] α MSH-ND was comparable to that of [^{6}His] α MSH-ND in the CHO cell lines stably transfected with hMC5R. It is possible that the relatively weak potency of these two α MSH analogues to MC5R might also have contributed to the result. MC5R selective analogues need to be developed to confirm this

issue.

In conclusion, there was no definite evidence of bone gain or loss after 4 weeks of subcutaneous injection of the αMSH analogues. Therefore, melanocortin might be applied clinically to humans without development of osteoporosis or osteopetrosis.

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