Genistein and menaquinone-4 treatment-induced alterations in the expression of mRNAs and their products are beneficial to osteoblastic MC3T3-E1 cell functions

MIDORI KATSUYAMA[1], MASASHI DEMURA[1], HIRONOBU KATSUYAMA[2], HIDEJI TANII[1] and KIYOFUMI SAIJOH[1]

[1]Department of Hygiene, Kanazawa University School of Medicine, Kanazawa, Ishikawa 920-8640; [2]Department of Public Health, Kawasaki Medical University, Kurashiki, Okayama 701-0192, Japan

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Abstract. The aim of the present study was to determine the molecular basis of the beneficial effects of genistein and/or menaquinone-4 (MK-4) on bone quality. Initially, 1 μM genistein was applied to MC3T3-E1 cells for 24 h and the upregulated mRNAs that were detected by microarray were selected for further examination by reverse transcription-quantitative-polymerase chain reaction. Among them, alterations were observed in the level of GATA-binding protein 6 (GATA6), Notch gene homolog 2 (NOTCH2), Wnt family member 5A (WNT5A), bone γ-carboxyglutamate protein (BGLAP), chondroadherin (CHAD), dipeptidyl peptidase 4 (DPP4), ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2), alkaline phosphatase (ALP) 3 and ATPase phospholipid-transporting 11A (ATP11A) in response to treatment with 0.1 μM 17-β-estradiol, 1 μM genistein, and/or 1 μM MK-4. GATA6, NOTCH2 and WNT5A are considered to be associated with osteoclast, but not osteoblast, function; however, increases in osteoblastic mRNAs, including BGLAP and CHAD, were observed in each of the treatment groups at 48 h. Immunocytochemical analysis confirmed an increase in CHAD and DPP4 proteins following the administration of genistein + MK-4. Furthermore, genistein + MK-4 led to alterations in cell morphology to spindle or oval shapes, and increased the intensity of ALP staining. Although the level of ALP mRNA was not consistently altered in response to the treatments, a marked increase in ALP activity was observed following 96 h treatment with genistein + MK-4. Therefore, the simultaneous intake of genistein and MK-4 appears to be beneficial for the maintenance of bone quality.

Correspondence to: Dr Kiyofumi Saijoh, Department of Hygiene, Kanazawa University School of Medicine, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8640, Japan
E-mail: saijohk@med.kanazawa-u.ac.jp

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Introduction

Bone is a basic component of the musculoskeletal system and is constantly remodeled by the function of osteoblasts and osteoclasts (1-3). This bone remodeling is maintained in men throughout life, whereas following menopause, women exhibit accelerated bone loss due to a decrease in estrogen levels, leading to osteoporosis (4). Osteoporosis is a serious social and health problem in a progressively aging society, and the associated increases in medical expenditure have become a serious problem in developed and developing countries (5). Various epidemiological studies have demonstrated that the intake of various vitamins and phytoestrogens can act to support the enrichment of bone mineral density (BMD) (6). Therefore, utilization of food-based nutrients is considered to be the most economical and simple way to prevent bone loss. For example, genistein, which has a similar structure to estrogen, has been demonstrated to aid in maintaining BMD (6-9). Furthermore, the simultaneous intake of genistein and menaquinone-7 (MK-7), a major form of vitamin K_2, is considered to be more effective for the maintenance of BMD (10-12). However, MK-7 is only present in fermented foods. For example, the traditional Japanese food, Nattō, a type of fermented soybean, is considered to be beneficial; however, due to its peculiar smell, its intake is not always common, even in Japan (10), and non-Japanese experience difficulty with its intake.

In addition to the extremely high concentration of MK-7 in Nattō (13), another major form of vitamin K_2, menaquinone-4 (MK-4) is found in meat, eggs and dairy foods; however, its concentration is often low (14,15). In addition, the bioavailability of MK-4 is unclear (16).

In the present study, the effects of genistein and/or MK-4 at dietary obtainable concentrations on the level of mRNAs and their protein products in MC3T3-E1 cells derived from neonatal mouse calvaria were evaluated.

Materials and methods

Reagents. Fetal bovine serum (FBS), and phenol red-free Eagle's minimal essential medium, α-modification (α-MEM), were obtained from Thermo Fisher Scientific, Inc. (Osaka,

Japan). Genistein, 17-β-estradiol and MK-4 were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany), and dissolved in ethanol (Nacalai Tesque, Inc., Kyoto, Japan).

MC3T3-E1 cell culture, treatment and RNA preparation. Osteoblastic MC3T3-E1 cells derived from the calvaria of a newborn C57BL/6 mouse were obtained from the Riken Cell Bank (Tokyo, Japan) and used at passages 3-5. The cells were maintained in α-MEM containing 10% (v/v) FBS. All cells were plated at a density of $1x10^5$ cells in 10-cm culture dishes, and incubated at 37˚C in a humidified atmosphere containing 5% CO_2. When subconfluent, the cells were subcultured into 35-mm dishes at a density of $1x10^5$ cells and then cultured with the media containing 0.1% ethanol (control), 0.1 μM 17-β-estradiol, 1 μM genistein, 1 μM MK-4, 17-β-estradiol + genistein (0.1 μM + 1 μM), 17-β-estradiol + MK-4 (0.1 μM + 1 μM) or genistein + MK-4 (1 μM + 1 μM) for 24, 48 or 96 h. The total RNA was extracted from 24 and 48 h cell cultures using ISOGEN reagent (Nippon Gene Co., Ltd., Tokyo, Japan). All total RNA samples were quality checked by RNA Pico Chips using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA), and treated with RNase-free DNase I recombinant (Roche Diagnostics GmbH, Mannheim, Germany) to isolate DNA-free RNA.

Identification of mRNA species upregulated by genistein. From each 24 h culture with or without 1 μM genistein, 1 mg total RNA was obtained. Oligo-dT (Sigma-Aldrich; Merck KGaA) purification was performed as previously described (17). Following this, polyA + RNA was compared using a mouse GE microarray version 2.0 (Agilent Technologies, Inc.). Among the mRNAs that were observed to increase by >3 times following treatment with genistein, 19 mRNAs with known functions (Table I) were subjected to reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in order to confirm the effects of 17-β-estradiol, genistein, MK-4, 17-β-estradiol + MK-4 and genistein + MK-4 at 24 and 48 h.

Quantification of mRNAs using RT-qPCR. From ~40 ng of total RNA, first-strand cDNA was synthesized using ReverTra Ace® reverse transcriptase (Toyobo, Co., Ltd., Osaka, Japan) with 5 pmol oligo-dT primers in a 20 μl reaction mixture, as indicated in the manufacturer's instructions, at 50˚C for 1.5 h. RT-qPCR was performed using the Stratagene Mx3000P qPCR System (Agilent Technologies, Inc.). The reaction mixture consisted of a final volume of 20 μl containing 1 μl of cDNA sample, 5 pmol of a set of gene-specific primers designed using Primer-3-Plus (http://primer3plus.com; Table I), and 10 μl of Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Inc.). The cycling conditions included a denaturing step at 95˚C for 2 min, followed by 60 cycles at 95˚C for 5 sec and 60˚C for 20 sec. The number of cycles of amplification required to reach the threshold (quantification cycle; Cq) were obtained using an amplification plot and the threshold line automatically reported. To determine the number of copies of the targeted mRNAs in the samples, the Cq values of the genes were normalized against that of β-actin using the $2^{-\Delta\Delta Cq}$ method (18). RT-qPCR products were run on 1% agarose gels and were confirmed as a single band.

Immunocytochemistry of chondroadherin (CHAD), dipeptidylpeptidase 4 (DPP4/CD26) and alkaline phosphatase (ALP). The protein expression levels of CHAD and DPP4 were immunocytochemically examined, with anti-ALP used as an MC3T3-E1 cell marker. The cells were seeded into BD Bio-Coat Collagen IV Cellware plates (Cosmo Bio Co., Ltd., Tokyo, Japan) at a density of $1x10^5$ cells/well, and incubated with 17-β-estradiol, genistein, MK-4, 17-β-estradiol + MK-4 or genistein + MK-4 for 48 or 96 h. The cells were fixed with 4% (w/v) paraformaldehyde in TBS for 30 min at room temperature. Subsequently, the cells were washed three times with 200 μl TBS and incubated in blocking solution containing 1% bovine serum albumin (Sigma-Aldrich; Merck KGaA) and 0.1% Tween-20 in TBS for 1 h at room temperature. Following a further wash with TBS, the fixed cells were incubated overnight at 37˚C with a goat polyclonal anti-ALP antibody (5 μg/ml; cat. no. AF2910; R&D Systems, Inc., Minneapolis, MN, USA), rabbit polyclonal anti-CHAD antibody (dilution, 1:500; cat. no. NBP1-87031, Novus Biologicals, LLC, Littleton, CO, USA) and a rabbit polyclonal anti-DPP4 antibody (dilution, 1:500; cat. no. sc-9153, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The cells in each well were then washed three times with TBS, and incubated with fluorescein isothiocyanate-conjugated anti-goat and tetramethylrhodamine-conjugated anti-rabbit secondary antibodies (dilution, 1:800; cat. nos. T6778 and F7367, respectively; Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. The cells were incubated with DAPI (Cell Signaling Technology, Inc., Danvers, MA, USA) for nuclear staining and were viewed under a BZ-8000 All-in-One Fluorescence Microscope (Keyence Corporation, Osaka, Japan).

Measurement of ALP activity. The activity of ALP was measured in $1x10^5$ MC3T3-E1 cells in 12-well culture plates after 96 h of treatment. The cells were fixed with 4% (w/v) paraformaldehyde in TBS for 30 min at room temperature and washed three times in TBS. The activity of ALP was measured by adding 0.25 mg/ml naphthol AS-BI phosphate (Sigma-Aldrich; Merck KGaA) for 1 h. Following a wash with TBS, ALP activity levels were measured via image capture using a Canon IXY 50S camera (Canon Inc., Tokyo, Japan) and intensity measurement using Adobe Photoshop version-20160113.r.355 x64 (Adobe Systems, Inc., San Jose, CA, USA).

Statistical analysis. All values are presented as the mean ± standard deviation. In order to compare the differences between the control and treatment groups, all groups were compared using one-way analysis of variance with Tukey's honest significant difference applied as a post hoc test (19). Statistically significant differences are indicated in each table. Analyses were performed using SPSS software (version 19; IBM SPSS, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

The half-maximal inhibitory concentration of genistein against 17-β-estradiol is reportedly 145 nM for estrogen receptor α and 8.4 nM for estrogen receptor β, and the two receptors are almost completely occupied by genistein at concentrations of

Table I. mRNAs with expression increased >3 fold in the microarray assay following 24 h treatment with 1 μM genistein were subjected to reverse transcription-quantitative polymerase chain reaction to validate the expression level change.

Accession no.	Gene name (genetic symbol)	Primer sequence	
		Forward	Reverse
NM_007432	Alkaline phosphatase 3, intestine, not Mn requiring (ALP3)	agaagctgcaataccacaac	atttggttgctgttggaact
NM_015804	ATPase, class VI, type 11A (ATP11A)	gacttgtgggtgtgtcgatg	ggaagagaactgggtggaca
NM_007541	Bone γ carboxyglutamate protein (BGLAP)	gggcagagagagaggacagg	acctgtgctgccctaaagc
NM_007557	Bone morphogenetic protein 7 (BMP7)	atggtggtatcgagggtgga	tctcctacccctacaaggcc
NM_013878	Calcium binding protein 2 (CABP2)	ggacccatcagctccacaaa	ccaggagtttgaccgagacc
NM_007689	Chondroadherin (CHAD)	tggataatgggagggaacg	aaatccccgaccaagaggt
NM_010074	Dipeptidylpeptidase 4 (DPP4)	gttctggggacaggcatc	ccagcacatctattcccaca
NM_015744	Ectonucleotide pyrophosphatase/ phosphodiesterase 2 (ENPP2)	gcaggtatgtcttgagggtca	ctcgggtgagggacatcg
NM_010258	GATA binding protein 6 (GATA6)	gcatttttgctgccatctg	aaccccgagaacagtgacc
NM_008398	Integrin α 7 (ITGA7)	cagatcgcatggcactttcg	atcctcagcacctctgggat
M21041	Microtubule-associated protein 2 (MAP2)	accaggatgccagatttggg	accttcctccatcctccctc
NM_144557	Myosin VIIA and Rab interacting protein (MYRIP)	ttcttcaggaccttggcact	agagctgctgctcctaccag
BC059256	Notch gene homolog 2 (NOTCH2)	agagagcgagggaagatgga	gagcacccatacctgacacc
NM_172766	Nuclear factor related to κ B binding protein (NFRKB)	cccttggagaccctgctaag	aaagcctcctggtcccttg
NM_008873	Plasminogen activator, urokinase (PLAU)	gtgttggcctttcctcggta	gtggcagtgtacttggagct
NM_173413	RAB8B, member RAS oncogene family (RAB8B), mRNA	ccacaggaatgaacgacca	gagagcaacgagcatttgtttt
NM_009446	Tubulin, α 3 (TUBA3)	cgtggtattgctcagcatgc	agtttgccatctacccagcc
NM_009521	Wingless-related MMTV integration site 3A (WNT3A)	ggtgtttctccaccaccatc	cgctcagctatgaacaagca
NM_009524	Wingless-related MMTV integration site 5A (WNT5A)	ccaaacagctgcaacacctc	cagaaccagccacttagggg
NM_007431	Alkaline phosphatase, liver/bone/kidney (ALPL)	ctacgcaccctgttctgagg	ggcgtccatgagcagaacta

1-10 μM (20). Thus, the effects of 0.1-100 μM of genistein on MC3T3-E1 cell growth were examined (Fig. 1). This revealed that 100 μM genistein inhibited cell growth at 24 and 98 h. Thus, in order to select mRNAs that may be involved in modifying osteoblastic function, mRNAs from cells treated with and without 1 μM of genistein were compared using microarray analysis at 24 h. Among the mRNAs from genistein-treated cells, almost 13,000 spots were revealed to exhibit a >3 fold higher signal compared with mRNAs from cells not treated with genistein. Of these, the mRNAs without any known function were removed, and those potentially affecting osteoblastic function (Table I) were selected and subjected to RT-qPCR in order to confirm their level of expression in cultures treated with 17-β-estradiol, genistein, MK-4, 17-β-estradiol + MK-4 or genistein + MK-4 for 24 or 48 h.

Among the identified factors, no significant difference was observed for Wnt family member 3A (WNT3A; Table II). At 24 h, all treated groups tended to exhibit increased GATA-binding protein 6 (GATA6) mRNA levels compared with the control; however, these differences were not significant. This effect was also observed at 48 h, and the GATA6 level was significantly increased genistein-treated cells compared with the other treatment groups at 48 h. At

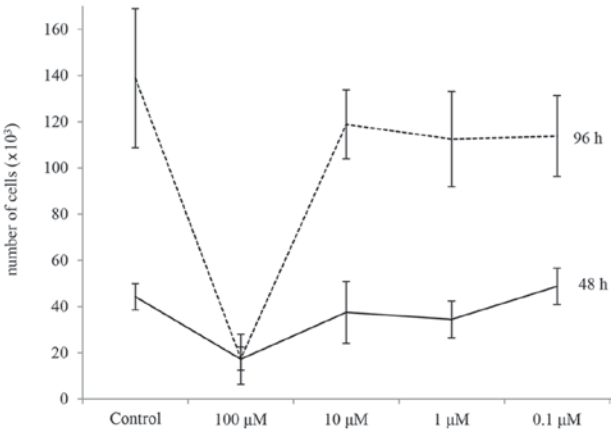

Figure 1. Proliferation of MC3T3-E1 cells with or without genistein. Values are presented as the mean ± standard deviation.

48 h, the levels of NOTCH2 and WNT5A in the genistein treatment groups tended to be higher compared with those of other groups, without statistical significance. NOTCH2 in the MK-4 treatment group was significantly lower than that in the genistein treatment group (P=0.0475).

Table II. Effects of 17-β-estradiol, genistein and/or MK-4 on mRNA levels of GATA6, NOTCH2 and WNT5A.

	GATA6		NOTCH2		WNT5A	
Treatment	Expression	P-value[a]	Expression	P-value[a]	Expression	P-value[a]
24 h						
Control	0.01±0.01	0.0018	0.12±0.14	0.049	0.33±0.46	0.0489
17-β-estradiol	10.92±6.05		2.45±1.40		3.37±5.10	
Genistein	1.53±2.83	0.0018	0.54±0.79		0.60±1.11	
MK-4	3.01±3.08	0.0066	0.04±0.10	0.0281	1.44±2.15	
17-β-estradiol+MK-4	12.60±29.74		0.45±0.26		0.27±0.36	0.0469
Genistein+MK-4	11.26±8.15		0.67±0.84		3.06±5.49	
48 h						
Control	0.75±0.73	0.0012	1.64±1.32		2.56±2.38	
17-β-estradiol	5.36±6.67	0.0097	3.09±4.66		6.94±9.09	
Genistein	34.20±27.60		5.58±6.61		13.16±17.21	
MK-4	4.18±3.35	0.0058	0.33±0.38	0.0475	3.58±3.29	
17-β-estradiol+MK-4	3.11±2.38	0.0037	2.01±1.24		3.71±3.15	
Genistein+MK-4	2.14±1.82	0.0024	3.06±3.30		5.68±4.38	

Values are presented as the mean ± standard deviation of 5-6 samples and expressed as 10^{-3}x β-actin expression. [a]P-value vs. genistein 48 h group (one-way analysis of variance with Tukey's honest significant difference test as a post hoc test). No significant difference in the expression of WNT3A was confirmed among any of the groups. GATA6, GATA binding protein 6; NOTCH2, Notch gene homolog 2; WNT5A, Wnt family member 5A; MK-4, menaquinone-4.

The levels of bone γ-carboxyglutamate (BGLAP) mRNA in all treatment groups, with the exception of genistein at 24 h, were increased compared with the control group, whether significant or not; the increase following genistein treatment at 48 h was statistically significant (Table III). 17-β-estradiol appeared to increase bone morphogenetic protein 7 (BMP7) mRNA at 48 h, although this difference was not significant compared with the 48 h control group. The levels of CHAD and DPP4 in all of the treatment groups at 48 h were higher compared with those of the control; however, a significant difference was observed only in the genistein treatment group. Similarly, the levels of ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2) mRNA tended to be increased following the treatments, however a significant difference was obtained only in the genistein treatment group at 48 h. A significant increase in ATPase phospholipid-transporting 11A (ATP11A) mRNA was observed following treatment with genistein + MK-4 for 48 h compared with the other groups. Differences in the expression levels of the following mRNAs were not observed between the treatment and control groups: Calcium-binding protein 2 (CABP2); nuclear factor related to κB-binding protein (NFRKB); integrin subunit α 7 (ITGA7); microtubule-associated protein 2 (MAP2); plasminogen-activator, urokinase (PLAU); RAB8B, member RAS oncogene family; and tubulin α 3 (TUBA3).

Alterations in CHAD and DPP4 mRNA levels were similar to each other (Table III). As these encode cell membrane-bound proteins that are suitable for quantitative analysis, the changes in their protein products were examined immunocytochemically (Fig. 2). At 48 h, an effect of 17-β-estradiol on CHAD or DPP4 was not always apparent. Genistein treatment induced the appearance of small fluorescent foci for CHAD and DPP4 along with alterations in cell morphology into oval or spindle-shape following 48 h treatment; such an effect was not always observed following MK-4 treatment. However, treatment with 17-β-estradiol + MK-4 induced the appearance of granular and diffuse CHAD and DPP4 staining, which were also apparent in the genistein + MK-4 group. After 96 h, alterations in the cell shape were marked in the MK-4 treatment cultures, which exhibited high intensities of CHAD and DPP4 staining. The small foci had disappeared, and larger spots along with diffuse staining appeared. These alterations were the most marked in Genistein + MK-4-treated cells.

A significant increase in ALP activity was observed only in the 96 h genistein + MK-4 treatment group compared with the other groups (Table IV). ALP3 mRNA was significantly increased in the 48 h genistein treatment compared with all other treatment and control groups (Table III), whereas alkaline phosphatase liver/bone/kidney mRNA exhibited no differences in expression in any of the treatment groups (data not shown).

Discussion

Among the Japanese population, the daily intake of genistein is reported to be ~12 mg (50 μmol) from 80 g of soy products (21), and people in eastern Japan typically consume 50 g of Nattō each day, supplying 3-5 mg of MK-7 (~6 μmol) simultaneously (22). Pre-menopause, Nattō intake is useful to promote bone formation (11). By contrast, MK-4 is contained in daily foods like meat, eggs and dairy foods, and can be consumed daily, but its concentrations are low at 1-10 nmol/100 g, thus, MK-4 intake is very limited (15). In the present study, in order to elucidate the utility of MK-4, mRNAs that exhibited altered levels in response to genistein treatment were identified initially in MC3T3-E1 cells using a microarray. However, microarrays are not always useful for quantitative analysis. Therefore, to validate the effect of genistein and/or

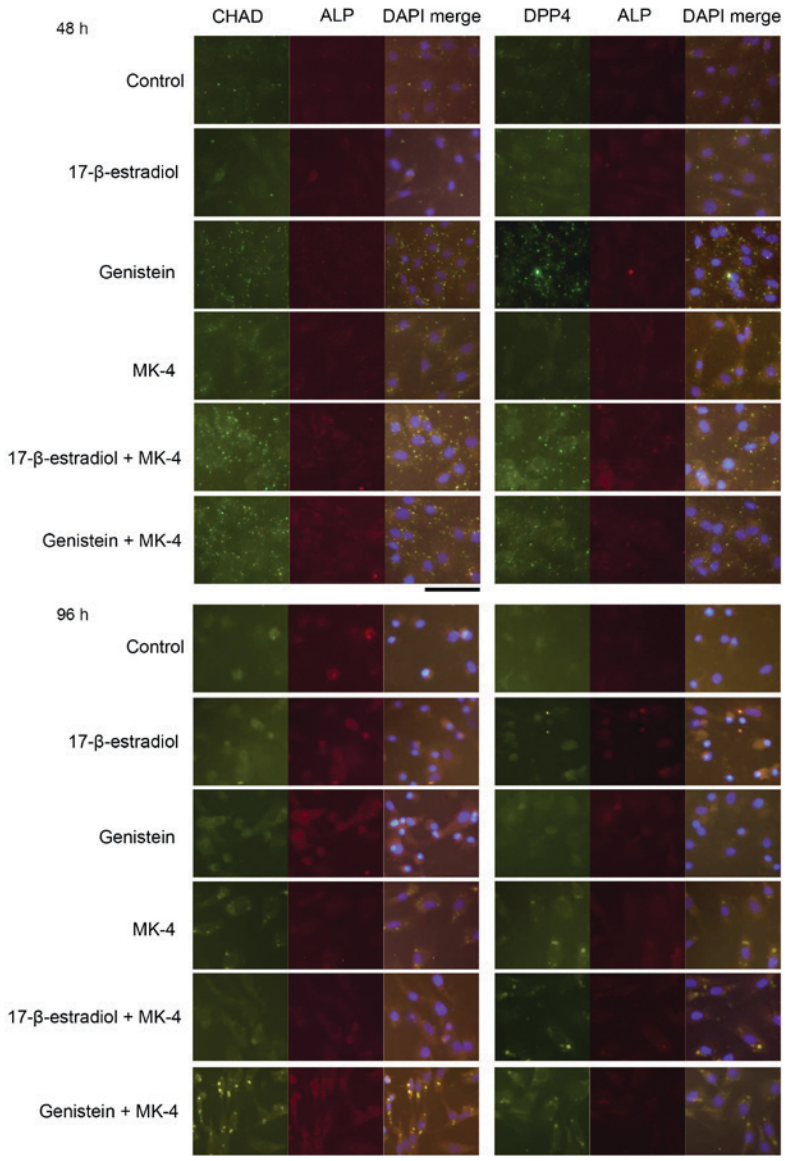

Figure 2. Immunocytochemical analysis of CHAD and DPP4 proteins in control cells and cells treated with 17-β-estradiol, genistein, MK-4, 17-β-estradiol + MK-4 or genistein + MK-4 for 48 and 96 h. Left panel, anti-CHAD or anti-DPP4 staining; center panel, anti-ALP staining; and right panel, merge with DAPI staining. Scale bar, 100 μm. MK-4, menaquinone-4; CHAD, chondroadherin; DPP4, pipeptidylpeptidase 4; ALP, alkaline phosphatase.

MK-4 on selected mRNAs, in comparison with the effects of 17-β-estradiol, RT-qPCR was also performed.

Although the mRNAs were selected because genistein increased their levels by >3 fold compared with the control at 24 h, this effect could not be confirmed in certain mRNAs. Genistein is a phytoestrogens that exerts similar effects to 17-β-estradiol, although these effects are not always identical. For example, at 48 h, a significant increase in GATA6 mRNA was observed following genistein treatment, but not following 17-β-estradiol treatment. However, the 17-β-estradiol + MK-4 and genistein + MK-4 maintained the expression level of GATA6 at a level consistent with that of the control group. As GATA6 is reported to suppress bone differentiation (23), the administration of estrogenic substances together with MK-4 appears to be beneficial for bone formation.

Osteoclastogenesis and bone resorption are inhibited by NOTCH1 and enhanced by NOTCH2 (24). In mouse embryonic stem cells, recombinant WNT5A has been reported to significantly enhance osteogenic yield, while recombinant WNT3A or other positive regulators of β-catenin decrease the expression of osteogenic markers (25). However, WNT5A is usually considered to promote osteoclast differentiation and prevent adipocyte differentiation (26). Genistein alone (27) and MK-4 alone (28,29) reportedly promote bone formation; however, in the current study, their co-administration appeared to be more beneficial as it allowed the maintenance of NOTCH2 and WNT5A mRNAs at the levels observed in the control cells.

In the current study, genistein and/or MK-4 treatments were shown to increase BGLAP, also known as osteocalcin, indicating that this promoted an osteoblastic phenotype in the MC3T3-E1 cells. In fact, cell morphology was altered following 96 h treatments, also exhibiting a high intensity of ALP. The increase of BMP7 by 17-β-estradiol also indicated beneficial

Table III. Effects of 17-β-estradiol, genistein and/or MK-4 on genes potentially associated with osteoblast function.

	BGLAP		BMP7		CHAD		DPP4		ENPP2		ALP3		ATP11A	
Treatment	Expression	P-value[a]	Expression	P-value[b]	Expression	P-value[a]	Expression	P-value[a]	Expression	P-value[a]	Expression	P-value[a]	Expression	P-value[c]
24 h														
Control	6.62±2.50	0.0021	0.02±0.02	0.0623	0.22±0.20	0.0037	0.46±0.37	0.0147	1.98±0.36	0.0066	0.26±0.17	0.0001	0.01±0.01	<0.0001
17-β-estradiol	71.34±55.19		0.25±0.32		2.98±1.20		9.23±8.42		16.26±11.69	0.0448	3.18±2.34	0.026	0.02±0.03	<0.0001
Genistein	5.67±3.00	0.002	0.03±0.04	0.0454	0.20±0.19	0.0016	0.79±0.91	0.009	1.96±0.40	0.0066	0.25±0.18	0.0001	0.01±0.02	<0.0001
MK-4	52.57±35.58	0.0304	0.30±0.51		2.70±2.41	0.1167	7.77±8.33	0.0385	9.58±5.62	0.019	2.08±1.95	0.0028	0.01±0.02	<0.0001
17-β-estradiol+ MK-4	84.84±190.21		0.02±0.03	0.0379	0.19±0.18	0.0101	0.17±0.13		45.99±106.03		0.38±0.31	0.0001	0.20±0.42	0.0003
Genistein+ MK-4	78.00±53.11		0.14±0.21		3.15±4.12	0.0419	10.28±13.17		25.47±17.67		3.19±2.59	0.0158	0.11±0.15	<0.0001
48 h														
Control	29.56±14.22	0.0085	0.17±0.22		0.34±0.58	0.0118	2.56±1.25	0.0223	7.16±1.70	0.0137	1.04±0.62	0.0005	0.16±0.16	0.0001
17-β-estradiol	108.22±66.35		0.72±0.86		0.98±0.09	0.0232	9.64±12.96		17.18±10.33		3.93±3.32	0.0445	0.02±0.02	<0.0001
Genistein	243.69±226.79		0.20±0.27		10.80±10.09		56.22±75.94		91.57±72.08		10.84±10.40		0.04±0.07	<0.0001
MK-4	77.27±38.57		0.25±0.35		1.40±1.39	0.0045	3.73±3.99	0.0169	19.88±7.95		2.23±1.39	0.0035	0.29±0.28	0.002
17-β-estradiol+ MK-4	56.23±26.86	0.0368	0.09±0.10		1.64±0.89	0.0105	3.83±1.41	0.0285	11.99±6.44	0.0261	2.26±1.28	0.0037	0.20±0.09	0.0003
Genistein+ MK-4	56.21±30.18	0.0368	0.09±0.10		1.24±0.64	0.0037	6.00±5.57	0.027	24.42±10.26		3.61±2.07	0.0287	0.94±0.66	

Values are presented as the mean ± standard deviation of 5-6 samples and expressed as 10-3x β-actin expression. [a]P-values vs. genistein 48 h group, [b]P-value vs. 17-β-estradiol 48 h group; [c]P-value vs. genistein+MK-4 48 h group (all groups were compared using one-way analysis of variance with Tukey's honest significant difference test as a post hoc test and significant comparisons are presented). No significant difference was confirmed among the groups in the expression of CABP2, ITGA7, MAP2, MYRIP, PLAU, RAB8B and TUBA3. BGLAP, bone γ-carboxyglutamate protein; BMP7, bone morphogenic protein 7; CHAD, chondroadherin; DPP4, pipeptidylpeptidase 4; ENPP2, ectonucleotide pyrophosphatase/phosphodiesterase 2; ALP3, alkaline phosphatase 3, intestine, not Mn requiring; ATP11A, ATPase class VI type 11A; CABP2, calcium binding protein 2; ITGA7, integrin α 7; MAP2, microtubule-associated protein 2; MYRIP, myosin VIIA and Rab interacting protein; PLAU, plasminogen activator urokinase; RAB8B, RAB8B, member RAS oncogene family; TUBA3, tubulin, α 3; MK-4, menaquinone-4.

Table IV. ALP activities at 96 h.

Treatment	ALP activity	P-value[a]
Control	44367±6992	0.0029
17β-estradiol	48205±8507	0.0175
Genistein	42629±1646	0.0013
MK-4	41318±1379	0.0007
17β-estradiol+MK-4	43170±2215	0.0017
Genistein+MK-4	62844±4095	

ALP activities were determined by photo and their intensity was measures by Photoshop. Thus, values are presented as the mean ± standard deviation of 5-6 samples and expressed by an arbitrary unit from Photoshop. [a]P-value vs. genistein + MK-4 group (one-way analysis of variance with Tukey's honest significant difference test as a post hoc test). ALP, alkaline phosphatase; MK-4, menaquinone-4.

effect of estrogenic stimulation on osteoblastic activity. The background of high activity of ALP by genistein + MK-4 was not always obvious.

Significant increases in the mRNA level of CHAD by MK-4 treatment were not consistently observed within 48 h, whereas strong expression of its protein was clearly visible by immunocytochemistry. CHAD has been reported to reduce preosteoclast motility and bone resorption without affecting osteoblast parameters, including the expression of runt related transcription factor 2 and BGLAP, the activity of ALP and bone formation (30). Therefore, the findings of the current study indicated a beneficial effect on bone formation in response to genistein + MK-4 treatment. However, the expression of DPP4 does not always appear to be beneficial for bone formation (31). Type 2 diabetes is associated with an increased risk of fracture. Incretins (gastric inhibitory polypeptide, glucagon like peptide 1, and 2) have an important role in the regulation of bone turnover and insulin release, and are digested by DPP4 (31). Therefore, DPP4 inhibitors appear to be useful for patients with type 2 diabetes, to ameliorate diabetes and also to prevent bone fracture. DPP4 is potentially induced by genistein and/or MK-4, of which intake is inevitable in healthy people via daily food consumption; however the concentrations obtained from food tend to be low.

ENPP2 encodes a secreted protein (32), which is therefore unsuitable for immunocytochemical analysis and was not assessed in the current study. Acidosis increases the mRNA levels of ENPPs and potentially contributes to decreased mineralization. However, among these ENPP proteins, the level of ENPP2 is not high in osteoblasts (33). ATP11A likely drives the transport of ions, such as calcium, across membranes (34); therefore, its increase by genistein + MK-4 treatment may be beneficial for the maintenance of bone.

In summary, the results of the present study suggest that the administration of genistein and/or MK-4 may be beneficial to maintain and/or improve BMD and bone metabolism. Compared with MK-7, the dietary intake of MK-4 is more common, despite its low concentration in foods. The simultaneous administration of MK-4 and genistein appears to have a substantial beneficial effect; thus, the consumption of beans with meat, eggs and dairy foods is advisable to increase osteoblastic activity.

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