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RESEARCH ARTICLES

Effect of genistein on the expression of bone metabolism genes in ovariectomized mice using a cDNA microarray

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Abstract

Osteoporosis associated with estrogen deficiency is defined as an abnormal decrease in bone mass leading to an increased fracture risk. Genistein (GEN), as a phytoestrogen, is a type of soybean-derived isoflavone that possesses structural similarity to estrogen. In this study, we assessed the effect of GEN in ovariectomized (OVX) mice. To determine the effect of GEN on bone metabolism, we investigated gene expression profiles using a radioactive cDNA microarray. Eight-week-old female mice were either sham operated (SHAM) or OVX. From 1 week after the operation, OVX mice were injected daily with intraperitoneal GEN (0.1, 0.5, 1.5 and 3.0 mg/day) or 17β-estradiol (E_2, 0.03 μg/day) for 4 weeks. A cDNA microarray was used to evaluate changes in the expression of 1,152 genes. OVX mice showed bone mineral density (BMD) loss versus SHAM mice (5.8±0.4 vs. 6.9±0.6 mg/cm^2). However, femur BMDs were completely restored by GEN and by E_2 administration in OVX mice. Serum osteocalcin in OVX mice treated with 0.5 mg/day of GEN was 1.6-fold (44.30±5.73 ng/ml) higher than that in untreated mice. GEN treatment up-regulated 38 genes (e.g., mitogen-activated protein kinase 10) and down-regulated 18 (e.g., matrix metalloproteinase 13). Moreover, GEN was found to have a protective effect on bone loss caused by estrogen deficiency in OVX mice. The present study suggests that GEN modulates bone metabolism-related gene expression, including calciotropic receptor, cytokines, growth factors and bone matrix proteins.

Keywords: Osteoporosis; Genistein; Bone metabolism; Gene profiling; cDNA microarray

1. Introduction

Osteoporosis is a metabolic disease of the bone and increases the likelihood of bone fracture. Decreased bone density is a major risk factor of osteoporosis [1,2]. The generalized loss of bone, the development of osteoporosis and the occurrence of fractures all increase with age. Whereas bone mineral density (BMD) declines in both men and women with age, women typically start with lower BMDs and show an accelerated loss at menopause owing to a decline in estrogen production by ovarian hormone deficiency [3]. It is well known that estrogen deficiency as in postmenopause and ovariectomy leads to acceleration of bone resorption and results in rapid bone loss with a high bone metabolic turnover, increasingly developing to osteoporosis [4]. Moreover, the occurrence of osteoporosis is associated with a large increase in osteoclast numbers caused by enhanced osteoclast formation and reduced osteoblast activity.

Current therapies recommended for postmenopausal osteoporosis treatment include supplementation with estrogen or hormone replacement therapies (ERT or HRT), calcitonin, bisphosphonates and raloxifene [5]. Estrogen is the most potent inhibitor of bone resorption and the most widely recommended therapy to reduce the rate of postmenopausal bone loss. However, available evidence

Abbreviations: OVX, ovariectomized; SHAM, sham operated; GEN, genistein; E_2, 17β-estradiol; Veh, Vehicle; BMD, bone mineral density.

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appears to suggest that the long-term use of ERT has numerous side effects (e.g., uterine bleeding and hyperplasia, cardiovascular disease, gall bladder disease, estrogen-induced endometrial cancer and an increased risk of breast cancer) [6].

Currently, natural alternatives to estrogen with estrogen-like activities such as soy isoflavone are being investigated as possible alternatives to estrogen for HRT [7–9]. Soy isoflavone is a phytoestrogen, which is a family of nonsteroidal molecules (isoflavones, lignans, coumestans, stilbenes and resorcyclic acid lactones) that occur naturally in a variety of plants [10]. The main classes of phytoestrogens are isoflavones, coumestans and lignans. In recent years, several reports have indicated that genistein (GEN), an isoflavone found predominantly in soy, prevents bone loss associated with estrogen loss in postmenopausal women [11–13] and experimentally in ovariectomized (OVX) animals [14,15].

The purpose of this in vivo study was to elucidate whether the administration of GEN, the most abundant and most active phytoestrogen in soy, is capable of preventing rapid bone loss occurring in female mice after surgical castration and, if so, to investigate the mechanism of this effect by using a cDNA microarray.

2. Materials and methods

2.1. Animals and chemicals

Eight-week-old female balb/c mice were obtained from Biogenomics (Seoul, Korea). All animals were housed under controlled lighting (12-h light/darkness) and temperature (21–22 °C) and maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. After 1 week of adaptation, 72 mice were randomized by weight into eight groups of eight animals, which were sham operated (SHAM, one group) or OVX (seven groups). Animals had access to food and water ad libitum.

After 1 week of adaptation after surgery, animals were fed a casein-base phytoestrogen-free diet (AIN-93G) and water, available ad libitum, starting 2 days before the treatment regimens were initiated. Some OVX mice were administered graded doses of GEN (Sigma, St. Louis, MO, USA) intraperitoneally dissolved in a solvent (20% dimethyl sulfoxide in corn oil) for 4 weeks daily. Other OVX mice received an intraperitoneal administration of 17β-estradiol (E_2, 0.03 μg/day, Sigma) dissolved in 20% dimethyl sulfoxide in corn oil. Control mice were treated with a vehicle (Veh) solution. In each experiment, body and uterus weights were measured. After 4 weeks, the tibiae and femora of mice were removed to measure BMD and to isolate total RNA (tRNA).

2.2. BMD measurement

Bone densitometry of whole femora was performed by X-ray absorptiometry (Model Discovery A S/N 80417, Hologic, USA) equipped with a high-resolution module for small-animal BMD evaluations. BMD was measured at baseline and at follow-up.

2.3. ELISA for osteocalcin

Serum osteocalcin was measured using a dual-antibody ELISA kit purchased from NAROO Ditech (Biomedical Technologies, USA); in brief, 25 μl of standard, sample or a control into a 96-well plate precoated with osteocalcin antibody. Osteocalcin antiserum was added to each well, and after incubation for 18–24 h at 2–8 °C, plates were washed with phosphate saline wash buffer. Then, 100 μl of streptavidin horseradish peroxidase reagent was added to each well and incubated for 30 min at room temperature. After washing plates thoroughly, 100 μl of a substrate solution (TMB solution/hydrogen peroxide solution=1:1) was added to each well and incubated in the dark for 15 min at room temperature; stop solution was then added. Osteocalcin concentrations were quantified by measuring absorbency at 450 nm.

2.4. cDNA microarray

Tibiae and femora were harvested, immediately frozen in liquid nitrogen and stored at −70°C until required. Frozen bones were homogenized using a Polytron homogenizer (PRO Scientific, Monroe, CT, USA). tRNA was isolated using TRIZOL, according to the protocol provided by the

Table 1
Dose-dependent effect of GEN on body and uterus weight in OVX mice

	Final body weight (g)	Percentage increase in body weight (initial to final, %)	Uterus weight (mg)	Uterus weight/ Body weight
SHAM ($n=8$)	23.12 ± 0.50^a	17	67.07 ± 2.01^a	2.92^a
OVX ($n=8$)	26.26 ± 0.99^b	31	34.25 ± 1.05^b	1.31^b
GEN 0.1 ($n=8$)	25.34 ± 1.14^b	27	40.19 ± 3.17^b	1.58^b
GEN 0.5 ($n=8$)	23.59 ± 1.67^a	17	31.44 ± 1.02^b	1.32^b
GEN 1.5 ($n=8$)	22.81 ± 1.54^a	13	28.13 ± 1.11^b	1.26^b
GEN 3.0 ($n=8$)	23.97 ± 2.34^a	16	37.21 ± 1.06^b	1.56^b
Veh ($n=8$)	23.19 ± 1.93^a	16	28.05 ± 1.22^b	1.24^b
E_2 ($n=8$)	23.85 ± 1.57^a	17	51.37 ± 2.14^a	2.15^a

Values with the same letters are not statistically different by Duncan's multiple-test one-way analysis of variance ($P\leq.05$). GEN 0.1 indicates OVX mice injected with GEN 0.1 mg/day; GEN 0.5, OVX mice injected with GEN 0.5 mg/day; GEN 1.5, OVX mice injected with GEN 1.5 mg/day; GEN 3.0, OVX mice injected with GEN 3.0 mg/day; and E_2, OVX mice injected with E_2 0.03 μg/day. Results are given as means±S.E.M.

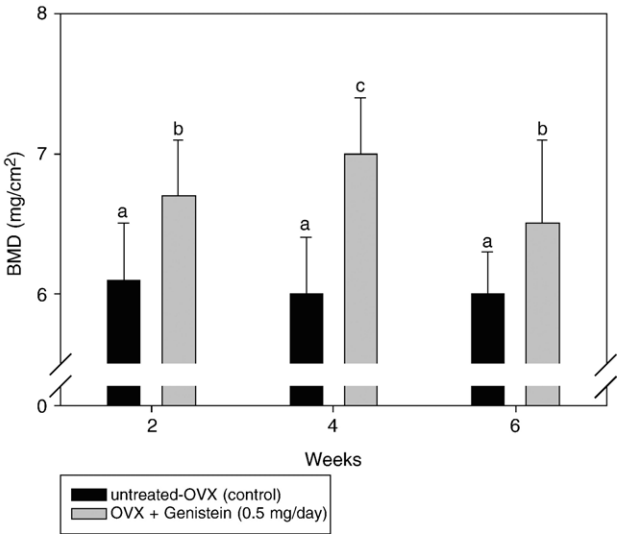

Fig. 1. Effect of GEN on the BMD of OVX mice across time to 6 weeks. The BMD of femur increased significantly by 2, 4 and 6 weeks. Data are expressed as the mean±S.E.M. of eight animals. Bars with the same letter are statistically not different by one-way analysis of variance. [b]Significantly different from untreated OVX mice: $P<.014$. [c]Significantly different from untreated OVX mice: $P<.0001$.

manufacturer (http://www.protocol-online.org/). tRNAs pooled from eight animals of each group were used for cDNA microarray analysis.

Polymerase chain reaction-amplified cDNAs were spotted on nylon membranes. Two nylon membrane mouse immunology arrays were used for the hybridization of each group. The general methodology of arraying was based on the procedures of DeRisi et al. [16]. cDNA microarrays were prehybridized in 50-ml Falcon tubes in a hybridization buffer containing 4.0-ml Microhyb (Research Genetics), 10 μl of 10-μg/ml human Cot 1 DNA (Life Technologies) and 10 μl of 8-mg/ml poly dA (Pharmacia, NJ, USA); both Cot 1 and poly dA were denatured at 95°C for 5 min prior to use. After 4 h of prehybridization at 42°C, approximately 10^7 cpm/ml of heat-denatured (95 °C, 5 min) probes was added, and the microarrays were then incubated for 17 h at 42 °C. The hybridized arrays were washed three times for 15 min in 2× SSC and 0.1% SDS at room temperature and exposed to phosphoimager screens for 1–3 days. The screens were then scanned in an FLA-8000 Phosphoimager (Fujifilm, Japan) at 50-μm resolution. Array images were opened, cropped and aligned in an L process V 1.96 (Science Lab). Aligned images were then opened in an ArrayGauge V 1.21 (Fujifilm, Inc, Japan) and a predefined grid was counted.

2.5. Statistical methods

Data analysis of microarray was used by EXCEL (Microsoft). To normalize each membrane or each gene across membranes, we used global normalization basic methods, which calculate the mean or median of the signal intensities of each individual experimental data set and then calculate the mean of the means (or grand mean) for all of the included experiments. Each individual data set is then mathematically adjusted such that the mean of that data set equals the calculated grand mean [17]. Raw intensity values obtained from the previous step were exported to EXCEL and normalized with Z transformation by subtracting with each average of gene intensity and dividing with each S.D. The Z score value represents the position of each gene intensity and provides us with the flexibility to compare between microarray experiments by adjusting for differences in hybridization intensities as follows:

$$Z\ \text{value}_{(\text{gene1})} = \log_{10}\left[\ \text{raw intensity}_{(\text{gene1})}\right] - \log_{10}\left[\ \text{mean raw intensity}\ _{(\text{all genes})}\right] / \text{S.D.}\log_{10}\left[\ \text{raw intensity}\ _{(\text{all genes})}\right]$$

$$Z\ \text{difference}_{(\text{gene1})} = Z_{(\text{gene1,array1})} - Z_{(\text{gene1,array2})}$$

$$Z\ \text{ratio}_{(\text{gene1})} = Z\ \text{difference}_{(\text{gene1})} / \text{S.D.}_{(Z\ \text{difference all genes})}$$

This facilitates comparison of each gene that had been up-regulated or down-regulated among different treatments or samples. Color overlay images were produced in an ArrayGauge V 1.21 (Fujifilm). Cluster analysis was performed on Z-transformed microarray data by using two programs available as shareware from Michael Eisen's

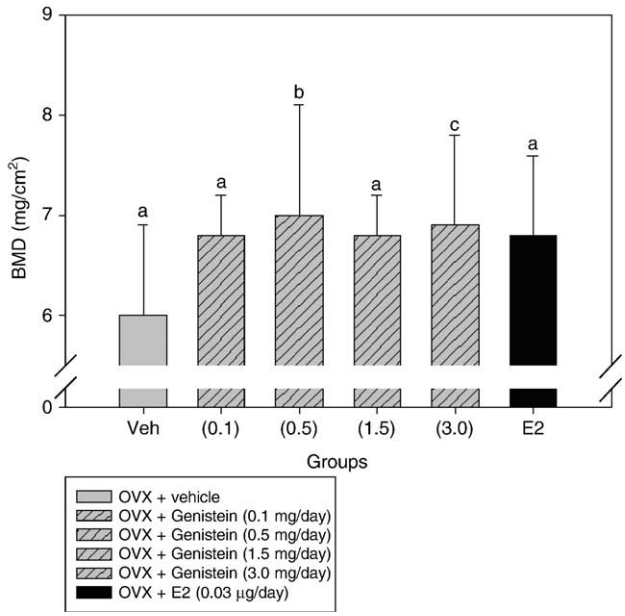

Fig. 2. Dose-dependent effects of GEN on BMD of OVX mice. Dose-dependent protective effect of intraperitoneal GEN injections on the BMD of femur in 8-week-old balb/c female mice 4 weeks after sham operation or ovariectomy. Data are expressed as the mean±S.E.M. of eight animals. Bars with the same letter are statistically not different by one-way analysis of variance. [b]Significantly different from untreated OVX mice: $P<.006$. [c]Significantly different from untreated OVX mice: $P<.003$.

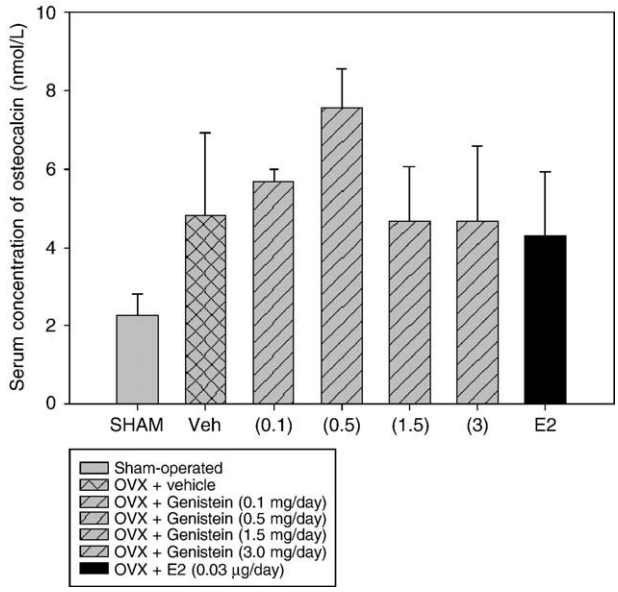

Fig. 3. Effect of GEN according to dose dependence in serum concentration of osteocalcin. Serum levels of osteocalcin in SHAM, untreated OVX and OVX mice treated with GEN or E_2. Serum was collected 4 weeks after the operation in SHAM, OVX and OVX mice treated with 0.1, 0.5, 2.5 and 3.0 mg/day of GEN or 0.03 μg/day of E_2, and the concentration of osteocalcin in each mouse was measured by ELISA as described under Materials and Methods. Data are expressed as the mean±S.E.M. of eight animals. Bars with the same letter are statistically not different by one-way analysis of variance. [b]Significantly different from untreated OVX mice: $P<.0001$. [c]Significantly different from untreated OVX mice: $P<.002$.

laboratory (http://rana.lbl.gov). Clustering of changes in gene expression was determined by using public domain cluster based on pairwise complete-linkage cluster analysis. Gene expression raw data, log values and Z scores were averaged by using the mean±S.D.

3. Results

3.1. Effects of GEN on body and uterus weight

All groups gained weight during the course of the experiment (i.e., 17% SHAM, 31% OVX, 27% OVX+GEN 0.1, 17% OVX+GEN 0.5, 13% OVX+GEN 1.5, 16% OVX+GEN 3.0, 16% OVX+Veh and 17% OVX+E_2 on initial body weight; Table 1). OVX mice showed a 14% greater weight gain than SHAM mice ($P<.05$), but this increase was not observed in OVX+GEN 0.1–3.0 mg/day mice. And treatment with GEN 0.5 mg/day and E_2 0.03 μg/day restored them to the SHAM level.

Uterus weights relative to the group body weights are presented in Table 1. It was observed that OVX mice showed a marked reduction in relative uterus weight versus SHAM mice. OVX treated with E_2 did not show a decrease in uterus weight, but those mice treated with GEN exhibited a similar degree of atrophy as the OVX mice. Although the administration of GEN 3.0 mg/day was associated with a trend toward a higher uterus weight, this was far lower than that shown by OVX mice treated with E_2.

3.2. Effects of GEN on femur BMD

To determine the time-dependent effect of GEN on bone, OVX mice were treated with GEN 0.5 mg/day for 2, 4 and 6 weeks. The BMDs of femora at 2, 4 and 6 weeks reached 6.7±0.4, 6.8±0.4 and 6.6±0.6 mg/cm^2, respectively, in GEN-treated OVX mice and were significantly higher than those of untreated OVX mice. The BMD levels in GEN-treated OVX mice were higher at 4 weeks than that at 2 and 6 weeks (Fig. 1). The mean BMD of whole femur was significantly lower in OVX mice than in SHAM mice (5.8±0.4 vs. 6.9±0.6 mg/cm^2, data not shown). Femur BMD was completely restored by GEN at 0.5 and 3.0 mg/day and by E_2 administration in OVX mice. To determine GEN dosages that affect the bone, OVX mice were administered various doses of GEN for 4 weeks. Mean BMD levels of the GEN 0.1, 0.5, 1.5 and 3.0 mg/day groups reached 6.5±0.9, 7.0±0.4, 6.8±1.1 and 6.9±0.4 mg/cm^2 or 6.8±0.8 mg/cm^2, respectively (Fig. 2). Daily injections with 0.5 mg of GEN prevented BMD loss 1.1-fold higher than that in the untreated OVX mice.

3.3. Serum levels of osteocalcin in OVX mice administered with GEN

The mean serum level of osteocalcin at 4 weeks was 4.84±2.08 nmol/L in OVX mice, which was higher than that of SHAM mice. In the 0.1 and 0.5 mg/day of GEN-treated groups, serum osteocalcin levels were 5.68±0.31 nmol/L

Table 2
Up-regulated gene expression in GEN-treated OVX mice by cDNA array

Gene	Z ratio[a]
Ubiquitin-conjugating enzyme E2M (homologous to yeast UBC12)	8.03
ESTs, weakly similar to epidermal growth factor receptor kinase substrate[b]	3.81
SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 5	3.80
Basic transcription factor 3	3.78
General transcription factor IIB	3.67
Sorting nexin 1[b]	3.26
Profilin 1[b]	3.21
One morphogenetic protein 4[b]	3.18
Glycogenin	3.15
Homeo box A4[b]	2.98
Protein tyrosine phosphatase, receptor type, alpha polypeptide	2.91
Prostate differentiation factor	2.80
Chloride channel, nucleotide-sensitive 1A[b]	2.59
MAPK 10[b]	2.57
Potassium voltage-gated channel, shaker-related subfamily, beta member 1	2.54
ESTs, moderately similar to RNA helicase HDB/DICE1 [*Homo sapiens*]	2.54

Z ratio$_{(gene1)}$, Z difference$_{(gene1)}$ and S.D.$_{(Z\ difference\ all\ genes)}$.

[a] Z ratio$_{(gene1)}$=Z difference$_{(gene1)}$/S.D.$_{(Z\ difference\ all\ genes)}$; fold of expression change for an individual gene based on the ratio for the GEN-treated probe compared with that of the untreated probe.

[b] Bone metabolism-related gene expression.

and 7.57±0.80 nmol/L, respectively, at 4 weeks. The administration of 0.5 mg/day of GEN resulted in a 1.6-fold higher serum osteocalcin concentration than that in untreated OVX mice, which suggests that a 1.6-fold elevation in the serum osteocalcin level may prevent bone loss caused by estrogen deficiency. Compared with untreated OVX mice, the 1.5 and 3.0 mg/day GEN-treated and the 0.03 μg/day of E_2-treated OVX mice showed lower serum levels of osteocalcin (Fig. 3).

3.4. Gene expression in GEN-treated OVX mice

Of the 1,152 genes in the cDNA microarray, gene expression analyses revealed that 38 genes were up-regulated and 18 were down-regulated by GEN 0.5 mg/day in OVX mice. Genes with a Z ratio ≥ 2 and those with a Z ratio ≤ -2 are listed in Tables 2 and 3. Genes showing highly altered expression levels in OVX mice treated with GEN were aligned in altered expression order. Fourteen of these up-regulated genes were bone metabolism related. Expressed sequence tags (ESTs, gene with unknown function), weakly similar to epidermal growth factor receptor kinase substrate and epidermal growth factor receptor pathway substrate 15, encode growth factor, and bone morphogenetic protein (BMP) 4 encodes bone matrix protein and sorting nexin 1, profilin 1, homeo box A4, chloride channel, mitogen-activated protein kinase (MAPK) 10, nucleotide-sensitive 1A, folylpolyglutamate synthase, gamma-aminobutyric acid A receptor delta, signal transducer and activator of transcription 1, catenin (cadherin-associated protein) alpha-like 1, tyrosine protein kinase (cAMP dependent, catalytic) inhibitor alpha and ribosomal protein S6 kinase polypeptide 4 encoding miscellaneous factors (e.g., genes associated with cellular signaling pathways). Seven of these down-regulated genes were bone metabolism related. Recoverin, protein tyrosine phosphatase, receptor type, alpha polypeptide,

Table 3
Down-regulated gene expression in GEN-treated OVX mice by cDNA array

Gene	Z ratio[a]
Human guanine nucleotide-binding regulatory protein (Go-alpha) gene	-3.64
X-ray repair complementing defective repair in Chinese hamster cells 1	-2.78
Recoverin[b]	-2.73
T-box, brain, 1	-2.68
Tumor necrosis factor (ligand) superfamily, member 10[b]	-2.65
NO DATA FOR THIS CLONE IN UNIGENE BUILD 106	-2.36
Protein tyrosine phosphatase, receptor type, alpha polypeptide[b]	-2.36
Nuclear receptor subfamily 1, group D, member 1	-2.32
ESTs, highly similar to endothelial NO synthase [*H. sapiens*][b]	-2.20
Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)[b]	-2.18
DKFZP434C128 protein	-2.13
ATP/GTP-binding protein	-2.11
A disintegrin and metalloproteinase domain 9 (meltrin gamma)[b]	-2.08
Laminin, beta 1	-2.05
Dynactin 1 [p150, Glued (*Drosophila*) homolog]	-2.05
Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)[b]	-2.03

[a] Z ratio$_{(gene1)}$=Z difference$_{(gene1)}$/S.D.$_{(Z\ difference\ all\ genes)}$; division of expression change for an individual gene is based on the ratio for the GEN-treated probe versus that of the untreated probe.

[b] Bone metabolism-related gene expression.

Table 4
Major candidate genes and their associations with bone metabolism in GEN-treated OVX mice by cDNA microarray

Biologic classification	Gene name	By GEN
Calciotropic receptor	Estrogen receptor 1	(+)
Cytokines	Interleukin 1, beta	(−)
	Interleukin 6 (interferon, beta 2)	(−)
	Tumor necrosis factor (ligand) superfamily, member 10	(−)
Growth factors	Insulin-like growth factor binding protein 2 (36 kD)	(+)
	Fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)	(+)
	Epidermal growth factor receptor pathway substrate 15	(+)
	Transforming growth factor, beta receptor II (70–80 kD)	(+)
Bone matrix proteins	BMP 4	(+)
	Collagen, type III, alpha 1	(−)
	A disintegrin and metalloproteinase domain 9 (meltrin gamma)	(−)
	MMP 13 (collagenase 3)	(−)
Miscellaneous factors	Catenin (cadherin-associated protein), alpha-like 1	(+)
	Signal transducer and activator of transcription 1	(+)
	Sorting nexin 1	(+)
	Gamma-aminobutyric acid A receptor, delta	(+)
	Ribosomal protein S6 kinase, 90 kD, polypeptide 4	(+)
	Chloride channel, nucleotide-sensitive 1A	(+)
	Tyrosine protein kinase inhibitor alpha	(+)
	Folylpolyglutamate synthase	(+)
	Protein tyrosine kinase 7	(−)
	ESTs, highly similar to endothelial NO synthase	(−)
	Protein tyrosine phosphatase, receptor type, alpha polypeptide	(−)
	Integrin, beta 5	(−)
	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	(−)
	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	(−)
	c-src tyrosine kinase	(−)
	MAPK 10	(+)
	Recoverin	(−)
	Profilin	(+)
	Homeo box A4	(+)
	Interferon-stimulated transcription factor 3 gamma (48 kD)	(+)

(+) indicates up-regulated gene by GEN; (−), down-regulated gene by GEN.

ESTs, highly similar to endothelial nitric oxide (NO) synthase [*H. sapiens*], integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor), integrin and alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor) encode miscellaneous factors (e.g., cellular signaling pathway genes), tumor necrosis factor (ligand) superfamily, member 10, encodes cytokine and a disintegrin and metalloproteinase domain 9 (meltrin gamma) encodes bone matrix protein. These genes clustered in Fig. 3 were grouped into several categories based on their bone metabolism by GEN (Table 4).

4. Discussion

The present study demonstrates that GEN, an abundant soybean isoflavone, prevents bone loss caused by estrogen deficiency without substantially affecting the uterus. OVX rat or mouse models are the most commonly used experimental animal models for studies on the mechanisms of osteoporosis and on the efficacies of potential therapeutic agents for the treatment and prevention of osteoporosis [18,19]. As expected, OVX mice in the present study exhibited all the characteristics of weight gain and uterine atrophy comparably not found in SHAM mice. However, OVX mice treated with GEN or E_2 appeared to gain weight to the SHAM level. Unlike estrogen, GEN had no effect on ovariectomy-induced uterus weight. Similar observations have been made by Ishimi et al. [20,21] although the dose of GEN used in their study was 1.7-fold higher. This weak uterotropic activity suggests that the effects of GEN on the uterus are different from those of estrogen. In our mouse model, the effect of GEN was quite different on the bone and uterus, which is the primary estrogen target organ. The intermediate dose of GEN (0.5 mg/day) produced maximal effect on bone but did not protect against loss of uterine size. These findings suggest that the beneficial effect of GEN on OVX mouse bone may be caused by mechanisms unrelated to the activation of estrogen receptor as the action of estrogen in nonclassical targets such as the brain, bone, cardiovascular system, kidneys, immune system and liver [22]. Setchell [23] suggests that arguments are made for considering soy isoflavones as natural selective estrogen receptor modulators based on recent data of their conformational binding to estrogen receptors. As such, this suggests that soy isoflavones are likely to have the beneficial effects of estrogen without the negatives, especially in tissues such as the endometrium and breast [24,25]. The possibility that the effect of GEN at 0.5 mg/day on OVX mice bone occurs via a nonestrogenic mechanism is supported by cDNA microarray expressional data. It was found that GEN can affect gene expression by interacting with cytokines, growth factor, bone matrix proteins and miscellaneous factors such as those cellular signaling pathway factors. Moreover, gene expression by GEN may be related to increased bone formation and decreased bone resorption (Table 4).

Also, serum osteocalcin was higher in OVX mice than in SHAM mice in this study. Osteocalcin is a protein that is secreted by mature osteoblasts and released during osteoclastic degradation and has been shown to be correlated with bone turnover rate [25], and serum osteocalcin concentrations were correlated with both active bone formation and resorption [25]. Moreover, obvious elevations in serum osteocalcin levels were found to be directly related to active bone formation [26,27]. Serum osteocalcin levels were well correlated with the amount of GEN administered to OVX mice. In fact, serum osteocalcin in OVX mice treated with GEN is indicative of the stimulatory effect of GEN on bone formation. This GEN response of the bone in OVX mice differs from that expected following E_2 administration, as most investigators have reported that the rate of bone formation in OVX rodents is suppressed rather than stimulated by E_2 [28,29]. Recent studies have suggested that increased bone resorption induced by estrogen deficiency in postmenopausal osteoporotic women is mediated by the increased paracrine production of bone resorbing cytokines [30]. Much evidence suggest that the decline in ovarian function with menopause is associated with spontaneous increases in serum proinflammatory cytokine levels [31]. In our experiment, we noted the down-regulation of cytokines such as interleukin 1, beta, interleukin 6 (interferon, beta 2) and tumor necrosis factor (ligand) superfamily, member 10, by GEN. These data suggest that a mechanistic link exists between the suppression of the production of these cytokines, increased osteocalcin and osteoblastic activity and higher BMD observed in GEN-treated OVX mice.

MAPK is activated by distinct signaling pathways, and one of these is mediated through a tyrosine kinase-type cell membrane receptor activated by growth factors such as insulin, IGF-I and EGF [32]. We noted an up-regulation of signal transducer and activator of transcription 1, MAPK 10, insulin-like growth factor binding protein 2 (36 kD) and epidermal growth factor receptor pathway substrate 15 by GEN. GEN may have affected gene expressions that affect several distinct pathways of bone cells.

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes, including collagenases, gelatinases and stromelysins. Collagenases cleave fibrillar collagens at a neutral pH and play an important role in matrix remodeling. We noted a down-regulation of MMP 13 (collagenase 3) by GEN in OVX mice.

BMPs are members of the TGF-β superfamily that were originally identified in bone-inductive extracts of demineralized bone [32]. In our experiment, after being treated with GEN, BMP 4 and transforming growth factor beta receptor II (70–80 kD) were up-regulated versus OVX mice, suggesting that GEN stimulates bone formation.

It is known that GEN acts as a tyrosine kinase inhibitor [33]. This suppressive role of GEN on bone-resorptive cells appears to be mediated through an intracellular calcium signaling pathway that includes the inhibition of protein kinase and tyrosine kinase. We demonstrated using a cDNA microarray that tyrosine protein kinase inhibitor alpha was up-regulated by GEN and protein tyrosine kinase 7 and that

protein tyrosine phosphatase, receptor type and alpha polypeptide were down-regulated by GEN. We cannot rule out the possibility that GEN directly inhibits osteoclastic bone resorption and thereby protects against bone loss caused by estrogen deficiency.

NO synthase is expressed by bone-derived cells, and whole cytokines are potent stimulators of NO production and high concentrations of NO inhibit the osteoblast lineage and directly regulate osteoclastic activity [34]. We noted a down-regulation of ESTs, in a manner highly similar to that of endothelial NO synthase by GEN in OVX mice. This down-regulation suggests that NO synthase was down-regulated by cytokine suppression.

In summary, our results suggest that GEN may improve bone mass through the promotion of bone formation and the prevention of bone resorption. These discoveries provide evidence that GEN may be mediated by regulation of bone-related genes for calciotropic hormones and receptors, cytokines, growth factors and receptors, bone matrix proteins and miscellaneous factors (e.g., cellular signaling pathway factor). It is known that gene expression profiling using cDNA microarray is a useful first step toward understanding the functions of these known or novel genes [17]. The information gained from analysis of bone-related genes will likely contribute to better understanding of pathological conditions such as osteoporosis. Also, GEN may become an additional viable therapy for osteoporosis caused by estrogen deficiency.

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