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ApoE Gene Deficiency Enhances the Reduction of Bone Formation Induced by a High-Fat Diet Through the Stimulation of p53-Mediated Apoptosis in Osteoblastic Cells

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ABSTRACT: Osteoblast apoptosis increased in the tibias of apoE$^{-/-}$ mice fed with a high-fat diet, decreasing bone formation. The expression of *p53* mRNA in marrow adherent cells increased. LDL or oxidized LDL increased apoptosis in the calvarial cells of apoE$^{-/-}$ mice. The increase in p53-mediated apoptosis is apparently related to a high-fat diet–induced osteopenia in apoE$^{-/-}$ mice.

Introduction: The effects of high-fat loading and the *apolipoprotein E* (*apoE*) gene on bones have not been elucidated. We hypothesized that *apoE* gene deficiency (apoE$^{-/-}$) modulates the effects of high-fat loading on bones.
Materials and Methods: We assessed this hypothesis using wildtype (WT) and apoE$^{-/-}$ mice fed a standard (WTS and ApoES groups) or a high-fat diet (WTHf and ApoEHf groups). The concentration of serum lipid levels and bone chemical markers were measured. Histomorphometry of the femurs was performed using μCT and a microscope. Bone marrow adherent cells from the femurs were used for colony-forming unit (CFU)-fibroblastic (CFU-f) assay and mRNA expressions analysis. The apoptotic cells in the tibias were counted. TUNEL fluorescein assay and Western analysis were performed in cultures of calvarial cells by the addition of low-density lipoprotein (LDL) or oxidized LDL.
Results: In the ApoEHf group, the values of cortical bone volume and trabecular and endocortical bone formation of the femurs decreased, and urinary deoxypyridinoline increased. Subsequent analysis revealed that the number of apoptotic cells in the tibias of the ApoES group increased, and more so in the ApoEHf group. The ratio of alkaline phosphatase–positive CFU-f to total CFU-f was decreased in the ApoEHf group. *p53* mRNA expression in adherent cells of the apoE$^{-/-}$ mice increased and had a significantly strong positive correlation with serum LDL. TUNEL fluorescein assay of osteoblastic cells revealed an increase of apoptotic cells in the apoE$^{-/-}$ mice. The number of apoptotic cells in the apoE$^{-/-}$ mice increased with the addition of 100 μg/ml LDL or oxidized LDL. The p53 protein expression in apoE$^{-/-}$ cells exposed to 100 μg/ml LDL or oxidized LDL increased.
Conclusions: We concluded that *apoE* gene deficiency enhances the reduction of bone formation induced by a high-fat diet through the stimulation of p53-mediated apoptosis in osteoblastic cells.
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Key words: apolipoprotein E, low-density lipoprotein, lipid peroxide, apoptosis, osteopenia

INTRODUCTION

The effect of lipid metabolism on bones has been shown in previous studies. For instance, inverse correlations were clinically observed between the levels of plasma total cholesterol and lumbar spine BMD[(1)] and those of plasma low-density lipoprotein (LDL) and BMD of the radius.[(2)] In vitro studies showed that oxidized LDL inhibited the differentiation of osteoprogenitors into osteoblasts,[(3,4)] and an atherogenic high-fat diet reduced bone mineralization in mice.[(5)] Thus, it could be suggested that impairments of lipid metabolism, such as high serum levels of LDL and/or oxidized LDL, may decrease bone mass by reducing bone formation.

The effect of apolipoprotein E (apoE) on bones, on the other hand, has been controversial. ApoE is a structural component in the triglyceride-rich lipoprotein particles including very LDL (VLDL) and chylomicrons and their

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remnants. The apoE component is a ligand for the LDL receptor, consisting of three isoforms including E2, E3, and E4,[6] and belonging to the family of LDL receptor-related proteins (LRPs).[7] Accordingly, deficiency of the *apoE* gene (apoE$^{-/-}$) in mice has a considerable effect on cholesterol transport; as a result, an increase in the plasma level of VLDL because of the failure of LDL receptor and LRP-mediated clearance from blood causes an increase in that of LDL.[8] The expression of the *apoE* gene has been observed in osteoblasts,[9] and some studies have shown that the apoE genotype is related to fracture risk and BMD.[10–14] However, others did not confirm this relationship.[15–18]

In rodents, the calcification of vessels did not occur in apoE$^{-/-}$ mice given a standard diet during the growth period.[8] When apoE$^{-/-}$ mice were given a high-fat diet, however, the vascular atherosclerotic lesions dramatically increased, as well as the plasma levels of LDL and VLDL.[19] These data indicate that the effect of apoE$^{-/-}$ on atherosclerosis in the vascular walls could depend on the amount of dietary fat given to the animals.

We hypothesized that apoE$^{-/-}$ modulates the effects of high-fat loading on bones. In this study, we examined the bone mass, turnover, and differentiation of osteogenic cells in four groups of mice, with or without the *apoE* gene, given a standard or high-fat diet during the age period of 5–17 wk. We also assessed the effects of apoE$^{-/-}$ and lipid loading on apoptosis of osteoblastic cells obtained from the calvariae of wildtype (WT) and apoE$^{-/-}$ mice.

MATERIALS AND METHODS

Animal preparation

Homozygous apoE$^{-/-}$ mice on a C57BL/6 background (N11) were obtained from Taconic (Hudson, NY, USA). Male mice homozygous for the apoE$^{-/-}$ were mated to female C57BL/6J mice (Clea Japan, Tokyo, Japan). Three hundred eggs of the subsequent heterozygous generation were collected, and after in vitro fertilization with heterozygous spermatozoa, the embryos were transferred into 50 foster mothers (ICR; Clea Japan), as described previously.[20] Using RT-PCR, the mice were genotyped at 3 wk of age to identify the homozygous apoE$^{-/-}$ and WT mice in the litter.

Experimental design

Thirteen male apoE$^{-/-}$ and 15 WT littermate mice were used in this study. Those mice were weaned at 4 wk of age and acclimatized for 1 wk. At the age of 5 wk, the mice were fed a standard diet (1.25% calcium, 1.06% phosphorus, 2.0 IU/g vitamin D_3; Clea Japan) or atherogenic high-fat diet (1.25% cholesterol, 7.5% cacao butter, 7.5% casein, 0.5% sodium cholate; Oriental Yeast Co., Tokyo, Japan), divided into four body weight-matched groups (WT mice fed with the standard diet, WTS group; WT mice fed with a high-fat diet, WTHf group; apoE$^{-/-}$ mice fed with the standard diet, ApoES group; apoE$^{-/-}$ mice fed with a high-fat diet, ApoEHf group) and maintained on the diet for 12 wk. Food intake was assessed as food weight (g) per mouse per day and matched among the four groups. All mice were housed individually in cages.

At the age of 17 wk, urine samples were collected for 24 h before death. After exsanguination, the bilateral humeri, femurs, and tibias were harvested under pentobarbital sodium anesthesia. Bone labeling with intramuscular injection of calcein (8 mg/kg body weight) was performed at 7 and 3 days before death in the mice used for histomorphometry. For examination of serum and urine, morphometry, analysis of apoptotic cells and CFU-f assay, seven apoE$^{-/-}$ mice (ApoES, $n = 3$; ApoEHf, $n = 4$) and nine WT mice (WTS, $n = 5$; WTHf, $n = 4$) were used. For serum LDL and lipid peroxides and extraction of mRNA, six apoE$^{-/-}$ mice (ApoES, $n = 3$; ApoEHf, $n = 3$) and six WT mice (WTS, $n = 4$; WTHf, $n = 2$) were used. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the University of Occupational and Environmental Health.

Examination of serum and urine

Serum levels of LDL, high-density lipoprotein (HDL), total cholesterol, osteocalcin, and calcium were determined by routine laboratory methods (Panapharm Laboratories, Kumamoto, Japan). Lipid peroxidize production in the serum was determined by measuring thiobarbituric acid-reactive substances (TBARS). The amount of TBARS was calculated based on the standard curve using malondialdehyde (MDA, 0–30 nmol) and expressed as nmoles of MDA per milliliter. Urinary deoxypyridinoline (D-pyr) levels were determined with an enzyme immunoassay kit (Pyrilinks-D; Metra Biosystems, Mountain View, CA, USA), and the results are expressed as nM/mM of creatinine.

Structural analysis using μCT

The trabecular bones of the distal metaphysis and the cortical bones of the mid-diaphysis of the right femurs were analyzed by the μCT system (μCT40; Scano Medical, Bassersdorf, Switzerland), as reported previously.[21] On 3D analysis of the distal and middle femurs, the tissue volume (TV, mm^3) and the trabecular and the cortical bone volume (BV, mm^3) were measured directly, and the trabecular and the cortical bone volume fraction (BV/TV, %) were calculated.

Bone histomorphometry

Undecalcified sections were obtained from the site of the mid-diaphysis and the distal metaphysis of the right femurs. The specimens were dehydrated and embedded in methylmethacrylate (MMA) after toluidine blue staining to yield 5-μm-thick sections. The sections were further stained with alkaline phosphatase (ALP)/TRACP-double staining (Kureha Special Laboratory Co., Tokyo, Japan). For each section, the area of the secondary spongiosa was measured. To exclude the primary spongiosa, the regions within 500 μm of the growth plate and one cortical shell-width of the endocortical surface were not measured. For the structural parameters of the distal femur, the trabecular BV/TV (%) and trabecular bone surface (BS, μm) were obtained. For

bone formation parameters, the bone formation rate (BFR/BS, %-μm/d) was obtained.[22,23]

Paraffinized 5-μm-thick section replicates of the right proximal tibias were stained for TRACP, and trabecular osteoblast surface (Ob.S/BS, %) and osteoclast surface (Oc.S/BS, %) were obtained, as described previously.[24]

For each section of the middle femurs, the total cross-sectional area (mm^2), bone marrow area (mm^2), and cortical bone area (mm^2) were measured, and cortical thickness (μm) was calculated. BFR/BS (%-μm/d) and eroded surface (ES/BS, %) were measured in the endocortical envelopes.

Analysis of apoptotic cells in vivo

The right tibial section replicates were examined for apoptosis by TUNEL staining[25] using the APO-BRDU-IHCTM kit (CHEMICON International, Temecula, CA, USA) in accordance with the manufacturer's instructions. For ALP activity, the serial sections were stained using an alkaline phosphatase kit (86-R; Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions.

At a magnification of ×400, ALP^+ and apoptotic cells were counted from five random visual fields regardless of the trabecular and endosteal surface area, except the regions within 300 μm of the growth plate.

Evaluation of bone marrow cells

Preparation of bone marrow cells: Bone marrow from the bilateral humeri and left femurs was flushed with a total of 6 ml of α-MEM (Nacalai tesque, Kyoto, Japan). To assay ALP^+ colony forming unit-fibroblastic (CFU-f) formation, bone marrow cells were plated at 1×10^6 cells/well in 6-well plates in α-MEM containing 10% FCS (GIBCO, New York, NY, USA), 10 mg/ml of streptomycin and 10,000 U/ml of penicillin mixed solution (Nacalai tesque), 1.25 U/ml of nystatin (Sigma-Aldrich), and 50 μg/ml of ascorbic acid (Sigma-Aldrich). The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air with a medium, which was changed at 2-day intervals.

ALP^+ CFU-f: On day 13 of culture, the CFU-fs were fixed and stained, using an alkaline phosphatase kit (86-R; Sigma-Aldrich). A colony comprising >50 cells was defined as CFU-f. We counted the total and ALP^+ CFU-fs with the culture dishes backlit and calculated the ratio of ALP^+ CFU-fs to total CFU-fs (ALP^+ CFU-f/total CFU-f).[26,27]

RNA isolation from adherent cells and first-strand cDNA synthesis

The remaining bone marrow cells for each of three samples per group (only the WTHf group had two samples) were cultured in the α-MEM for extraction of total RNA. On day 3 of culture, the nonadherent cells were discarded, and the adherent cells were scraped with 1 ml of TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) and collected. The RNA was isolated from the adherent cells in accordance with the manufacturer's instructions and cleaned up using DNase I (Roche Applied Science, Penzberg, Germany). First-strand cDNA was reverse-transcribed from total RNA (1 μg) using Moloney murine leukemia virus reverse transcriptase (SuperScript; Invitrogen) and Oligo(dT)12–18 Primer (Invitrogen).

Quantitative RT-PCR

Quantitative RT-PCR analysis was performed using an iCycler apparatus (Bio-Rad Laboratories, Hercules, CA, USA) associated with the Icycler Optical System Interface software (version 3.1; Bio-Rad). The quantitative RT-PCRs for *type I collagen* (*col1a1*), *osteocalcin*, *p53*, *bax*, *bcl-2*, and *β-actin* were performed in 20 μl with ~7.5 ng cDNA, 0.5 μM of primers, and 10 μl iQ SYBR Green Supermix (Bio-Rad). PCR products of mouse *col1a1* (170 bp), *osteocalcin* (231 bp), *p53* (232 bp), *bax* (173 bp), *bcl-2* (183 bp), and *β-actin* (639 bp) were amplified using primers for *col1a1* (5′-acgtcc tggtgaagttggtc-3′ and 5′-cagggaagcctctttctcct-3′), *osteocalcin* (5′-tgacaaagccttcatgtcca-3′ and 5′-tgccagagtttggctttagg-3′), *p53* (5′-agagaccgccgtacagaaga-3′ and 5′-ctgtagcatgggcat ccttt-3′), *bax* (5′-tgcagaggatgattgctgac-3′ and 5′-gatcag ctcgggcactttag-3′), *bcl-2* (5′-ctggcatcttctccttccag-3′ and 5′-ga cggtagcgacgagagaag-3′), and *β-actin* (5′-ttgagaccttcaacac cccag-3′ and 5′-acttgcgctcaggaggagcaa-3′). These primers were designed using Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and synthesized at the Sigma-Aldrich Japan K.K. Genosys Division (Hokkaido, Japan). *β-actin* was used as an internal control. The amplification conditions were an initial 3 min at 95°C, 45 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s. Only *β-actin* was annealed at 65°C for 30 s. The mRNA expression levels were normalized with *β-actin* mRNA expression and expressed as relative value (fold change) to the expression of the WTS group, respectively.

Analysis of apoptotic cells in vitro

Preparation of osteoblastic cells from 19.5-day fetal calvariae of the respective genotypes, as described previously,[28] was performed. The cells were seeded at 2×10^4 cells/well (48-well plates) and cultured for 24 h, and subsequently, either human LDL (Athens Research & Technology, Athens, GA, USA) or human oxidized LDL (Biomedical Technologies, Stoughton, MA, USA) was added to the fresh medium at different concentrations (0, 10, 30, 60, and 100 μg/ml). TUNEL assay was performed using the In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science) according to the manufacturer's instructions, at the 24-h time-point, and the cells were counted at 100-fold magnification. The results are expressed as the ratio of apoptotic cell numbers to osteoblast numbers per microscopic field.

Western blot analysis

The osteoblastic cells were seeded at 2×10^5 cells in 6-cm dishes and cultured for 24 h, and subsequently, either 100 μg/ml human LDL or oxidized LDL was added to the fresh medium. The cells were lysed in lysis buffer and harvested at 0- and 24-h time-points. Aliquots equivalent to 2×10^4 cells were subjected to SDS-PAGE on 10% polyacrylamide

TABLE 1. BODY WEIGHT, SERUM LIPID LEVELS, AND BONE CHEMICAL MARKERS IN WT AND apoE$^{-/-}$ MICE FED WITH A STANDARD OR A HIGH-FAT DIET

	WT		*ApoE$^{-/-}$*	
	Standard diet (WTS)	*High-fat diet (WTHf)*	*Standard diet (ApoES)*	*High-fat diet (ApoEHf)*
Body weight (g)	25.2 ± 2.2	27.6 ± 2.8	23.5 ± 3.7	24.0 ± 2.1
Serum				
Total cholesterol (mg/dl)	102 ± 5.8	312 ± 34	1195 ± 155*	2443 ± 407†‡
LDL (mg/dl)	7.2 ± 2.8	84.7 ± 16	258 ± 29*	727 ± 124†‡
Lipid peroxides (nmol MDA/ml)	83.6 ± 27	56.4 ± 0.8	1210 ± 125*	1649 ± 173†‡
HDL (mg/dl)	47.6 ± 3.9	51.5 ± 5.1	10.0 ± 0.0*	6.7 ± 2.9†
Osteocalcin (ng/ml)	121.4 ± 32	72.8 ± 19	111 ± 11	65.7 ± 31
Calcium (mg/dl)	8.4 ± 0.5	8.2 ± 0.5	7.8 ± 0.2	8.2 ± 0.8
Urine				
D-pyr (nM/mM Cre)	14.4 ± 3.0	18.7 ± 2.9	11.2 ± 0.3	38.9 ± 2.6†‡

Results are expressed as mean ± SD over all animals in each diet group. These data are 17-wk-old mice.
* $p < 0.05$ vs. WT on standard diet.
† $p < 0.05$ vs. WT on high-fat diet.
‡ $p < 0.05$ vs. apoE$^{-/-}$ on standard diet.

gel and transferred to a PVDF transfer membrane (Hybond-P; GS Healthcare Bio-Science Corp., Piscataway, NJ, USA). The membrane was blocked with 5% nonfat milk in PBS containing 0.1% Tween 20 for 1 h at room temperature. p53 antibody (Sigma-Aldrich) was used. The membrane was incubated overnight at 4°C with the primary antibody diluted to 1:200. After washing, the membrane was incubated with the appropriate second antibody conjugated to horseradish peroxidase. Protein was detected by the ECL Plus Western Blotting Detection System (GS Healthcare Bio-Science Corp.).

Statistical analysis

All values are expressed as mean ± SD. They were assessed by the Tukey-Kramer posthoc test after one-way ANOVA. In the study of apoptotic cell analysis in vitro, statistical analysis was done by Student's *t*-test and Dunnet's method on the WT control group and Dunnet's method on the apoE$^{-/-}$ control group. Correlation studies were done using Pearson's correlation coefficient. Two-factor factorial ANOVA was used to detect the potential effects of apoE$^{-/-}$ and a high-fat diet. Statistically significant differences were set at <0.05. Calculation was performed with Stat View 5.0 software (SAS Institute, Cary, NC, USA) on a Macintosh computer.

RESULTS

Body weight, serum lipid levels, and bone chemical markers

No significant differences in body weight were found among the four groups at the end of the experiment, at 17 weeks of age after being given either a standard or high-fat diet for the duration of 12 weeks (Table 1). In the apoE$^{-/-}$ mice fed with the standard diet (ApoES), the values of total cholesterol, LDL and lipid peroxides significantly increased, compared with those in the WT mice fed with the standard diet (WTS). The values further significantly increased in the apoE$^{-/-}$ mice fed with a high-fat diet (ApoEHf), compared with those in the WT mice fed with a high-fat diet (WTHf) and the ApoES group. The levels of serum HDL in the ApoES and ApoEHf groups significantly decreased compared with those in the WTS and WTHf groups, respectively. Serum osteocalcin and calcium levels did not significantly differ among the four groups of mice. The values of urinary D-pyr significantly increased in the ApoEHf group compared with those in the WTHf and ApoES groups (Table 1).

Morphological and morphometrical assessments of bone mass and turnover in the femurs

Trabecular BV/TV in the distal femur and cortical BV/TV in the midfemur were measured by μCT (Fig. 1). The values of trabecular BV/TV did not significantly differ among the groups. However, the values of cortical BV/TV in the ApoEHf group were significantly smaller than those in the WTHf group (Table 2).

In the samples of mid- and distal femur, histological and histomorphometrical assessments under light microscopy were also performed on undecalcified sections of the specimens after fluorescence labeling. The values of cortical thickness of the mid-femur in the ApoEHf group were significantly smaller compared with the respective values in the WTHf and ApoES groups. The BFR/BS values in the trabecular bone surface in distal femoral metaphysis were reduced significantly in the ApoEHf group compared with the WTHf group (Table 2). In the samples of the proximal tibias, the trabecular Ob.S/BS values decreased, and the trabecular Oc.S/BS values increased in the WTHf and ApoEHf groups insignificantly (Table 2). The BFR/BS values in endosteum at the mid-femur were also reduced in the ApoEHf group. The ES/BS values in the ApoEHf group

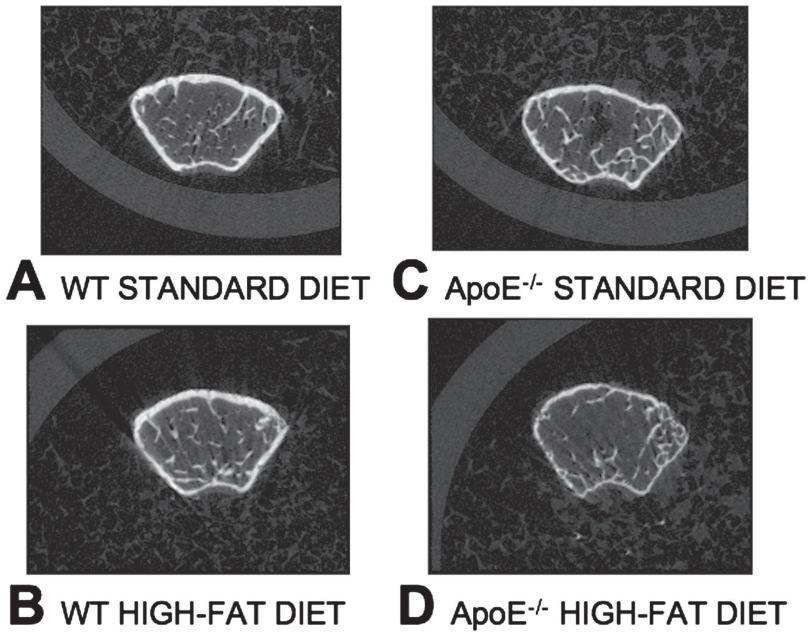

FIG. 1. 2D axial image of the middle femur from 17-wk-old mice using μCT in each diet group (A–D). Cortical thickness of the apoE$^{-/-}$ mice (C and D) decreased compared with the WT mice (A and B).

TABLE 2. MORPHOLOGICAL AND MORPHOMETRICAL ASSESSMENTS OF BONE MASS AND TURNOVER IN THE FEMURS

	WT		*ApoE$^{-/-}$*	
	Standard diet (WTS)	*High-fat diet (WTHf)*	*Standard diet (ApoES)*	*High-fat diet (ApoEHf)*
Bone mass				
Distal femur trabecular BV/TV (%)	5.94 ± 0.7	4.15 ± 0.9	6.21 ± 1.0	3.92 ± 1.6
Mid-femur cortical BV/TV (%)	29.8 ± 1.7	30.9 ± 1.9	25.9 ± 1.6	24.2 ± 2.2*
Cortical thickness (μm)	137.3 ± 7.5	136.3 ± 4.1	133.5 ± 5.6	112.5 ± 6.5†
Bone turnover				
Trabecular bone				
BFR/BS (%-μm/d)	41.6 ± 7.1	40.5 ± 2.3	32.2 ± 4.2	20.1 ± 5.5*
Ob.S/BS (%)	21.2 ± 4.2	12.7 ± 3.7	21.9 ± 11.6	8.8 ± 2.6
Oc.S/BS (%)	10.7 ± 5.1	14.9 ± 4.7	8.1 ± 1.2	15.1 ± 2.7
Cortical endosteum				
BFR/BS (%-μm/d)	59.5 ± 17.6	49.2 ± 6.9	32.8 ± 11.4	4.3 ± 7.5*
ES/BS (%)	3.28 ± 1.4	7.49 ± 4.5	4.75 ± 2.2	12.3 ± 1.9†

Results are expressed as mean ± SD over all animals in each diet group. These data are 17-wk-old mice.
* $p < 0.05$ vs. WT on high-fat diet.
† $p < 0.05$ vs. apoE$^{-/-}$ on standard diet.

significantly increased compared with those in the ApoES group (Table 2).

Alkaline phosphatase stain and TUNEL stain of bone and bone marrow

As reductions in bone formation and bone mass by a high-fat diet and apoE$^{-/-}$ were confirmed, we proceeded to evaluate the osteogenic cells in the bones and bone marrow of the mice. The number of ALP^{+} cells in the sections of decalcified specimens of the proximal tibia did not significantly differ among the four groups of mice (data not shown). However, the number of TUNEL^{+} cells in the bone and bone marrow significantly increased in the ApoES and ApoEHf groups compared with the WTS and WTHf groups, respectively (Figs. 2A–2E). The percent ratios of the number of TUNEL^{+} cells to ALP^{+} cells also significantly increased in the groups of apoE$^{-/-}$ mice compared with those in the groups of WT mice (Fig. 2F).

Assessments of bone marrow cells for total CFU-f and ALP^{+} CFU-f

To evaluate the bone marrow capacity for developing osteogenic cells, we assessed the numbers of total CFU-f and ALP^{+} CFU-f from bone marrow cells of the femur. The number of total CFU-f in the WTHf and ApoEHf groups was apparently smaller than the respective values of those in the WTS and ApoES groups, but the differences were not statistically significant (Fig. 3A). The ratios of ALP^{+} CFU-f to total CFU-f in the ApoEHf group were significantly smaller than those of the ApoES group (Fig. 3B).

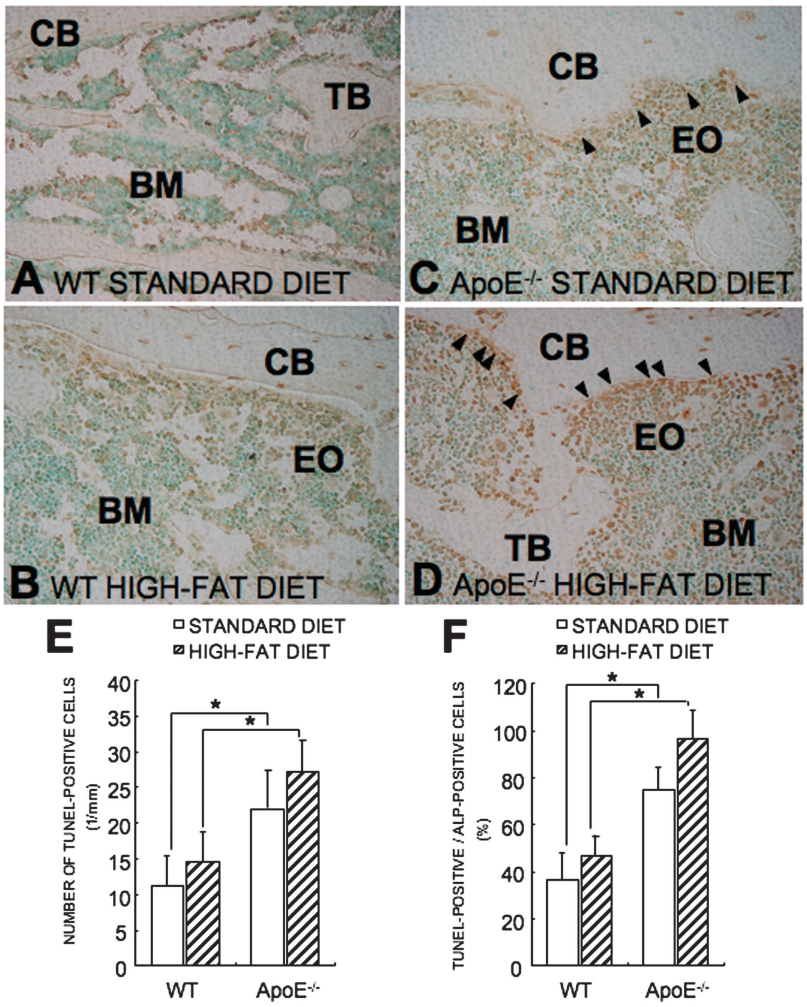

FIG. 2. TUNEL$^+$ cells in the proximal tibias (magnification, ×400). TUNEL stain showed an increase of apoptotic cells (arrowheads) at the endosteum in the apoE$^{-/-}$ mice (C and D) compared with the WT mice (A and B). BM, bone marrow; CB, cortical bone; EO, endosteum; Tb, trabecular bone. Cell quantification using serial sections in the proximal tibias. (E) The number of TUNEL$^+$ cells significantly increased in the ApoES and ApoEHf groups compared with the WTS and WTHf groups, respectively. (F) The ratio of TUNEL$^+$ cells to the ALP$^+$ cells also significantly increased in the ApoES and ApoEHf groups compared with the WTS and WTHf groups, respectively ($*p < 0.05$).

Assessments of mRNA expression of osteocalcin, type I collagen, p53, bax, and bcl-2 genes in bone marrow cells

Increases of apoptotic cells in ALP$^+$ cells in histology and reductions of ALP$^+$ CFU-f developments in bone marrow cells prompted us to study the mRNA expressions of genes relating to the development of osteogenic cells and apoptosis in bone marrow cells of the femur. We measured the mRNA expression of *osteocalcin*, *col1a1*, *p53*, *bax,* and *bcl-2* genes. The expression of *osteocalcin* mRNA did not significantly differ among the groups (data not shown). The expression of *col1a1* mRNA did not significantly differ either (Fig. 4A). The expression of *p53* mRNA in the ApoES and ApoEHf groups significantly increased compared with those in the WTS and WTHf groups, respectively (Fig. 4B). The expression of *bax* mRNA (data not shown) and the ratio of *bax* to *bcl-2* mRNA expression (Fig. 4C) had upward trends in the ApoES and ApoEHf groups, and the expression of *bcl-2* mRNA had a downward trend in the ApoES and ApoEHf groups (data not shown), but the differences were not statistically significant.

Statistical evaluations for the effect of a high-fat diet and apoE$^{-/-}$ on the development and apoptosis of osteogenic cells in bone marrow cells

The contributions of a high-fat diet and apoE$^{-/-}$ to the development and apoptosis of osteogenic cells in bone marrow cells were evaluated by two-factor factorial ANOVA. It showed that a high-fat diet significantly affected all the parameters of bone marrow CFU-fs, but apoE$^{-/-}$ did not significantly relate to these parameters of bone marrow cells (Table 3). Both a high-fat diet and apoE$^{-/-}$ significantly affected the ratios of the numbers of TUNEL$^+$ cells to ALP$^+$ cells. The expression of *osteocalcin* mRNA was not affected by either a high-fat diet or apoE$^{-/-}$. The expression of *col1a1* mRNA, however, was significantly affected by apoE$^{-/-}$. The expression of *p53* mRNA was significantly affected by both of these factors (Table 3).

Between the expression of *p53* mRNA in bone marrow cells and serum levels of LDL and lipid peroxides, significant linear correlations were found in apoE$^{-/-}$ mice, combined with the ApoES and ApoEHf groups (Figs. 5A and 5B). However, these correlations were not significant in the groups of WT mice, WTS and WTHf (data not shown). We

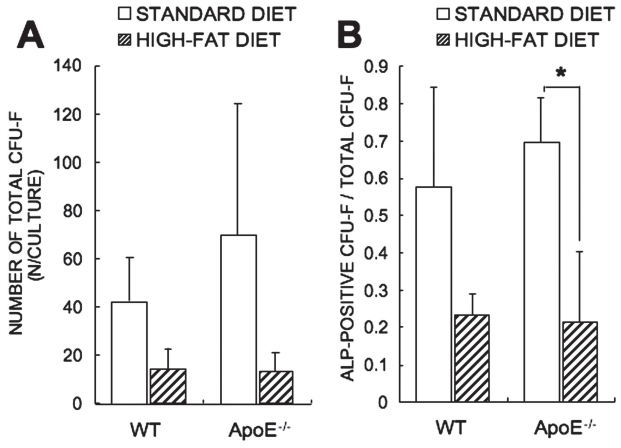

FIG. 3. The results of CFU-f assay using bone marrow cells. (A) The number of total CFU-f in the WTHf and ApoEHf groups was apparently smaller than the respective values of those in the WTS and ApoES group, but the differences were not statistically significant. (B) The ratio of ALP^+ CFU-f to total CFU-f significantly decreased in the ApoEHf group compared with the ApoES group ($*p < 0.05$).

calculated the ratio values of mRNA expression of *osteocalcin* to *col1a1* (mRNA *osteocalcin*/mRNA *col1a1*) as the parameter of the signals representing the capacity of developing osteogenic cells in bone marrow cells. A significant inverse relationship was found between the values of *p53* mRNA expression and the ratio values of mRNA *osteocalcin*/mRNA *col1a1* (Fig. 5C).

TUNEL fluorescein assay and Western blot analysis in cultures of osteoblastic cells obtained from fetal mice calvaria by the addition of LDL or oxidized LDL

To compare the effects of LDL and oxidized LDL on the development of apoptosis between WT and $apoE^{-/-}$ osteoblastic cells, we obtained fetal calvarial cells. In the cells from WT fetal mice, TUNEL fluorescein assays revealed a minimum increase in apoptotic cells in the culture media with ascending doses of LDL or oxidized LDL (Figs. 6A–6C). In the cells from $apoE^{-/-}$ fetal mice, however, the number of apoptotic cells was significantly larger than that of WT cells in the medium without LDL or oxidized LDL (Figs. 6D–6F) and significantly increased with the addition of 100 μg/ml LDL or lipid or oxidized LDL (Fig. 6G). Two-factor factorial ANOVA showed that the ratios of apoptotic cells in the culture media to which was added LDL or oxidized LDL were significantly affected by the respective lipid concentrations and $apoE^{-/-}$ as well (Table 4).

To examine the protein level of p53 in osteoblastic cells, we performed Western blot analysis. The p53 expression in the $apoE^{-/-}$ cells exposed to 100 μg/ml LDL or oxidized LDL at 0 and 24 h increased compared with that in the WT cells (Fig. 4D).

DISCUSSION

Our study showed that a high-fat diet and $apoE^{-/-}$ synergistically increased apoptosis in osteoblastic cells, leading to decreased trabecular and endosteal bone formation in mice. *p53* mRNA expression significantly increased in the two groups of $apoE^{-/-}$ mice compared with those in the respective groups of WT mice. There were significant linear correlations between the expression of *p53* mRNA in adherent stromal cells and serum levels of LDL and lipid peroxides. In the $apoE^{-/-}$ mice, the ratio of the numbers of ALP^+ CFU-f to total CFU-f significantly decreased in the group of a high-fat diet compared with that in the group of standard diet. Thus, it was revealed that, in the $apoE^{-/-}$ mice, the osteoblastic cells were vulnerable to apoptosis induced by either LDL or oxidized LDL.

Several previous studies have reported that bone volume and bone strength decrease in animals given a high-fat diet.[29–31] A high-fat diet reduces intestinal absorption of calcium and causes a negative balance between the intake and the output of serum calcium.[32,33] There is no difficulty in thinking that the deficient state of serum calcium is compensated for by osteoclastic bone resorption. Thus, we considered that a high-fat diet increases bone resorption consequent to a negative balance of calcium.

The number of ALP^+ cells in vivo did not significantly differ among the four groups of mice. However, the percent ratios of the number of $TUNEL^+$ cells to ALP^+ cells in vivo increased in the WTHf and ApoEHf groups compared with the WTS and ApoES groups, respectively (Fig. 2F). These in vivo data on histomorphometry indicate that the disappearance of ALP^+ cells increased in the WTHf and ApoEHf groups. Thus, we think that the apparent reductions in ALP^+ CFU-f assays in vitro (Fig. 3B) are consistent with the histomorphometry data in vivo in these groups. Also, we found that the BFR/BS and Ob.S/BS values decreased in the ApoEHf group (Table 2). These data are compatible with the reduction in life span of functional osteoblasts in vivo. All these data in vitro and in vivo consistently indicate that the apoptotic disappearance of osteoblastic lineage cells was enhanced in the condition of the deleted *apoE* gene under high-fat loading.

A previous study showed that the degree of hyperlipidemia correlated with the degree of osteoclastic functional activity in the marrow.[34] In our study, the $apoE^{-/-}$ mice fed with a high-fat diet revealed excessive hyperlipidemia; as a result, it increased bone resorption, excessively, compared with $apoE^{-/-}$ mice fed with the standard diet. On the other hand, another previous study suggested that increased dietary lipids interfered with osteoblast maturation and reduced bone mineralization in vivo,[5] and our results of trabecular BV/TV and serum osteocalcin are compatible with this previous study. Consequently, in the $apoE^{-/-}$ mice fed with a high-fat diet, bone resorption increased, and bone formation decreased remarkably, compared with the $apoE^{-/-}$ mice fed with the standard diet. Thus, we considered that the abnormal bone phenotype is only observed in $apoE^{-/-}$ mice fed with a high-fat diet, because of the uncoupling of bone resorption and formation.

p53 mRNA expression increased in adherent stromal cells by either a high-fat diet or $apoE^{-/-}$. The ratio of the *osteocalcin* mRNA to the *col1a1* mRNA negatively correlated with *p53* mRNA expression. TUNEL stain revealed that $apoE^{-/-}$ and a high-fat diet increased ALP^+ apoptotic

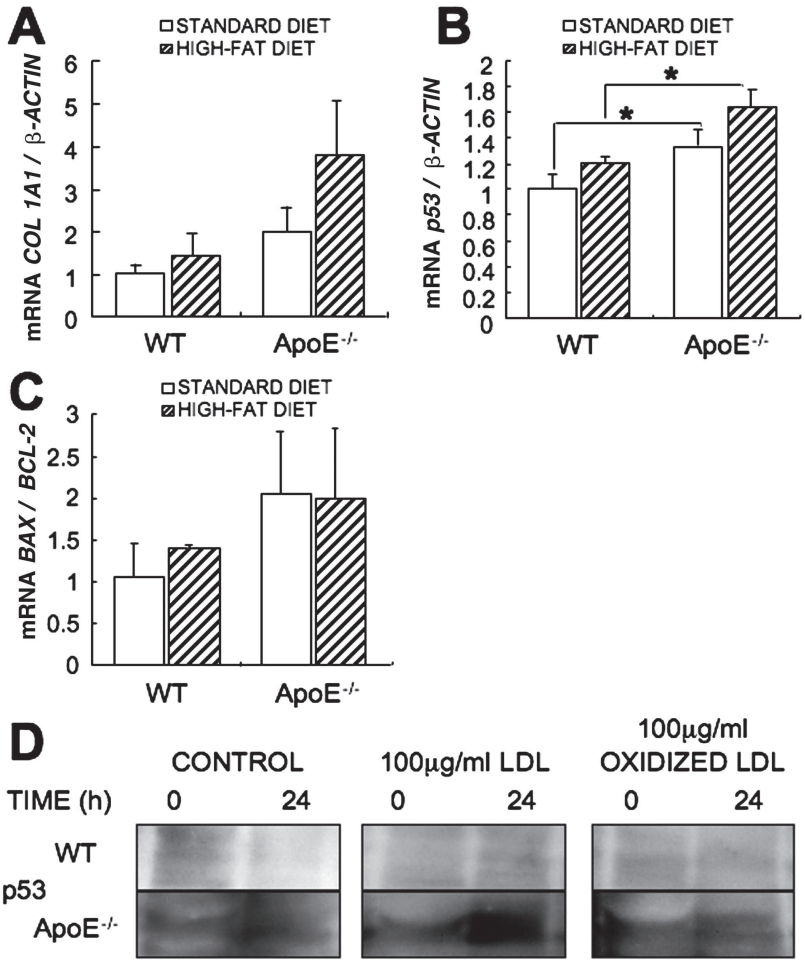

FIG. 4. Evaluation of mRNA expression in bone marrow adherent cells. (A) The expression of *col1a1* mRNA had an upward trend in the ApoEHf group, but the difference did not achieve statistical significance. (B) The expression of *p53* mRNA in the ApoES and ApoEHf groups significantly increased compared with those in the WTS and WTHf groups, respectively ($*p < 0.05$). (C) The ratio of *bax* to *bcl-2* mRNA expression had upward trends in the ApoES and ApoEHf groups. (D) Western blot analysis for p53 in cultures of osteoblastic cells with the addition of 100 μg/ml LDL or oxidized LDL. The p53 expression in the apoE$^{-/-}$ cells exposed to 100 μg/ml LDL or oxidized LDL at 0 and 24 h increased compared with that in the WT cells.

TABLE 3. STATISTICAL EVALUATIONS FOR THE EFFECT OF A HIGH-FAT DIET AND APOE$^{-/-}$ ON THE DEVELOPMENT AND APOPTOSIS OF OSTEOGENIC CELLS IN BONE MARROW CELLS

	ApoE $^{-/-}$	*High-fat diet*
Bone marrow cells		
Total CFU-f	0.427	0.008*
ALP$^+$ CFU-f	0.345	0.015*
ALP$^+$/total	0.588	0.003*
Proximal tibia		
TUNEL$^+$/ALP$^+$	<0.0001*	0.013*
Bone marrow adherent cells		
osteocalcin mRNA	0.156	0.968
col1a1 mRNA	0.01*	0.059
p53 mRNA	0.001*	0.01*

Data represent *p* values for the relationship. The significant differences were determined by two-factor factorial ANOVA and set at $p < 0.05$.
* Significant.

cells. Apoptosis in osteoblasts and osteoblast precursors was caused by a high-fat diet and further enhanced by apoE$^{-/-}$. It was previously reported that LDL or oxidized LDL caused apoptosis in endothelial cells,[35,36] vascular smooth muscle cells (VSMCs),[37,38] and osteoblastic Saos2 cells[39] and that apoE$^{-/-}$ also induced apoptosis of VSMCs[40] and neurons.[41] Although *p53* mRNA expression in the adherent stromal cells did not statistically correlate with serum LDL or lipid peroxides in the WT mice, they significantly correlated with it in the apoE$^{-/-}$ mice. Thus, the *apoE* gene in osteoblastic cells could prevent apoptosis induced by LDL or lipid peroxides. Those synergistic apoptotic actions of apoE$^{-/-}$ and LDL or lipid peroxides were obvious in the calvarial osteoblastic cell study. A high-fat diet and/or apoE$^{-/-}$ caused apoptosis in osteoblastic cells, and this could be a major reason for decreased bone formation in trabecular bone and endosteum.

In our study, apoE$^{-/-}$ mice fed with the standard diet could maintain constant levels of serum osteocalcin, the numbers of ALP$^+$ cells in vivo, and the numbers of ALP$^+$ colonies in vitro despite the increases of osteoblastic cell apoptosis. We measured the Ob.S/BS values, finding that the parameter apparently reduced under high-fat loading in both the WT and apoE$^{-/-}$ mice. These data suggest that the reduction in the number of bone-forming cells, mediated by apoptosis, was induced by high-fat loading, and the effects were enhanced in the *apoE* gene deleted condition. Regarding the serum osteocalcin levels, we also found that the ALP$^+$ cells in vivo and signal for bone-forming cell lineages such as mRNA *col1a1* in vitro did not decrease in the face of high-fat loading (Fig. 4A). Thus, we can assume that the recruitment of bone-forming cells was not reduced in either

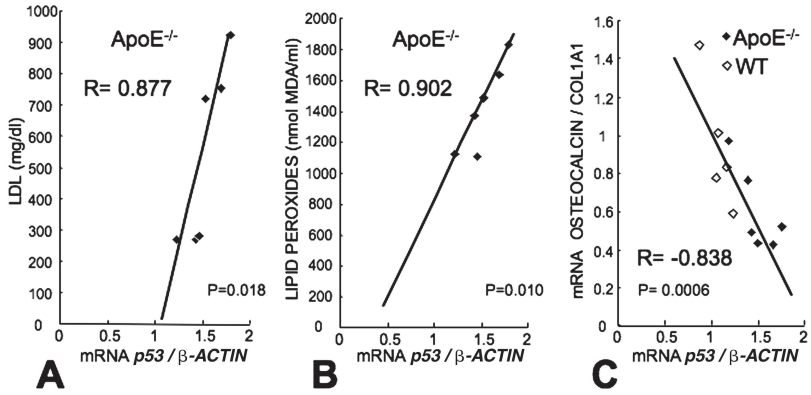

FIG. 5. Correlation between the expression of *p53* mRNA and lipid levels and the ratio values of mRNA expression of *osteocalcin* to *col1a1*. The expression of *p53* mRNA in the apoE$^{-/-}$ mice had a significantly strong correlation with serum LDL levels (A) and a much stronger correlation with serum lipid peroxide levels (B), but not in the WT mice (data not shown). (C) Correlation between the mRNA *osteocalcin* / mRNA *col1a1* ratio and the *p53* mRNA expression in bone marrow adherent cells had a significantly strong negative correlation. r = Pearson's correlation coefficient.

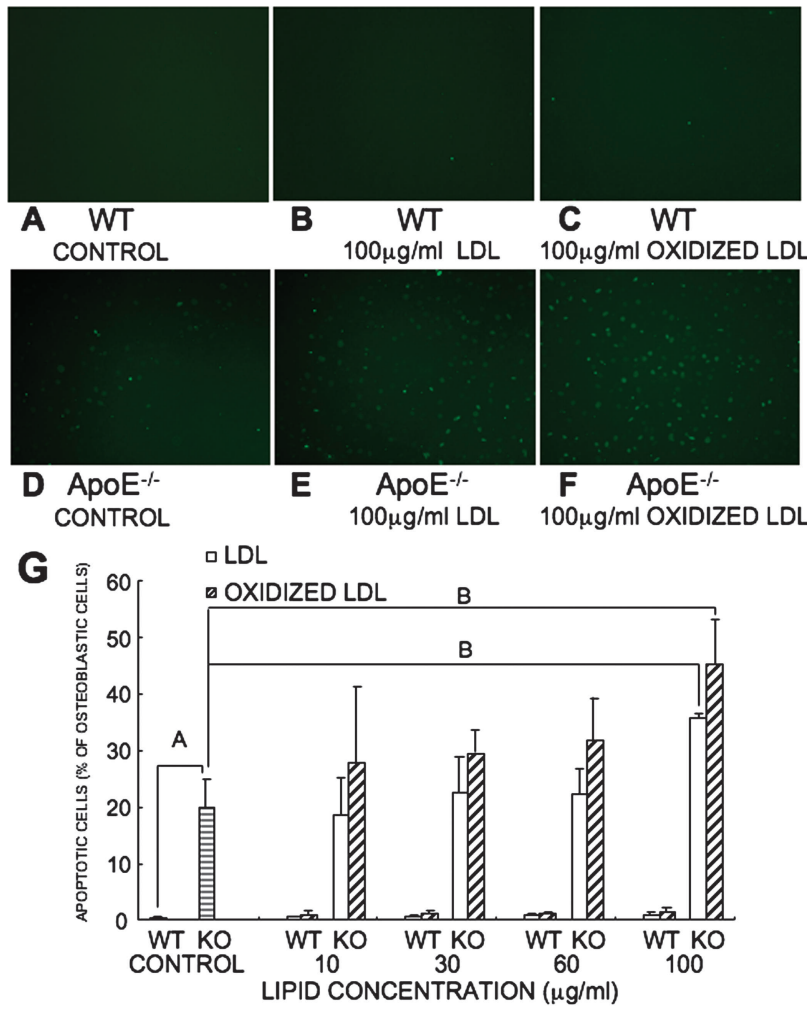

FIG. 6. Effects of the addition of LDL and oxidized LDL in cultures of osteoblastic cells from fetal mice calvaria using TUNEL fluorescein assay. Representative fluorescence micrographs of osteoblastic cells (A–F; magnification, ×100). In the cells from WT mice, there was a slight increase in apoptotic cell number by the addition of LDL and oxidized LDL (A–C). In the cells from the apoE$^{-/-}$ mice, in contrast, there were apoptotic cells in the apoE$^{-/-}$ control (D), and the number of apoptotic cells increased with addition of both 100 μg/ml LDL (E) and 100 μg/ml oxidized LDL (F). Quantification of the absolute number of apoptotic cells expressed as a percentage of the total number of osteoblastic cells. The percentage of apoptotic cells in the apoE$^{-/-}$ control significantly increased compared with that in the WT control. Addition of 100 μg/ml LDL and 100 μg/ml oxidized LDL in the apoE$^{-/-}$ significantly increased the percentage of apoptotic cells compared with the apoE$^{-/-}$ control. $^{A}p < 0.05$ compared with the WT control. $^{B}p < 0.05$ compared with the apoE$^{-/-}$ control.

group in this experiment, maintaining serum osteocalcin levels in the face of an apparent reduction in bone formation surface in vivo.

Apoptosis of osteoblastic cells has been reported as a possible mechanism of bone loss in a few kinds of disorders, such as rheumatoid arthritis,[42] glucocorticoid-induced osteoporosis,[43] and unloading-induced osteoporosis.[26,44] The mechanism of osteoblast apoptosis involves the modulation of mitochondrial functions, intracellular reactive oxygen species, and Bcl-2 protein.[45] Recently, it has been reported that high plasma homocysteine, which has been associated with an increased risk of fracture, enhances apoptosis in human bone marrow stromal cells through the intracellular reactive oxygen species–mediated mitochondrial pathway and NF-κB activation.[46] Our report is the first to describe that osteoblast apoptosis induced by lipid

Table 4. Statistical Evaluations for the Effect of Lipid Concentrations or apoE$^{-/-}$ in Cultures of Osteoblastic Cells From Fetal Mice Calvaria

Ratio of apoptotic cells	*ApoE$^{-/-}$*	*Lipid concentration*
Addition of LDL	<0.0001*	0.003*
Addition of oxidized LDL	<0.0001*	0.016*

Data represent p values for the relationship. The significant differences were determined by two-factor factorial ANOVA and set at $p < 0.05$.

* Significant.

loading through p53 signal is related to the reduced bone formation in apoE$^{-/-}$ mice.

This study suggests that the *apoE* gene prevented a high-fat diet from inducing apoptosis in osteoblasts and osteoblast precursors. In conclusion, *apoE* gene deficiency enhances the reduction of bone formation induced by a high-fat diet through the stimulation of p53-mediated apoptosis in osteoblastic cells.

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REFERENCES

1. Stulc T, Ceska R, Horinek A, Stepan J 2000 Hyperlipoproteinemia, the Apo-E genotype and bone density. Cas Lek Cesk **139:**267–271.
2. Yamaguchi T, Sugimoto T, Yano S, Yamauchi M, Sowa H, Chen Q, Chihara K 2002 Plasma lipids and osteoporosis in postmenopausal women. Endocr J **49:**211–217.
3. Parhami F, Morrow AD, Balucan J, Leitinger N, Watson AD, Tintut Y, Berliner JA, Demer LL 1997 Lipid oxidation products have opposite effects on calcifying vascular cell and bone cell differentiation. A possible explanation for the paradox of arterial calcification in osteoporotic patients. Arterioscler Thromb Vasc Biol **17:**680–687.
4. Parhami F, Jackson SM, Tintut Y, Le V, Balucan JP, Territo M, Demer LL 1999 Atherogenic diet and minimally oxidized low density lipoprotein inhibit osteogenic and promote adipogenic differentiation of marrow stromal cells. J Bone Miner Res **14:**2067–2078.
5. Parhami F, Tintut Y, Beamer WG, Gharavi N, Goodman W, Demer LL 2001 Atherogenic high-fat diet reduces bone mineralization in mice. J Bone Miner Res **16:**182–188.
6. Weisgraber KH, Rall SC Jr, Mahley RW 1981 Human E apoprotein heterogeneity. Cysteine-arginine interchanges in the amino acid sequence of the apo-E isoforms. J Biol Chem **256:**9077–9083.
7. Herz J, Beffert U 2000 Apolipoprotein E receptors: Linking brain development and Alzheimer's disease. Nat Rev Neurosci **1:**51–58.
8. Zhang SH, Reddick RL, Piedrahita JA, Maeda N 1992 Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. Science **258:**468–471.
9. Bachner D, Schroder D, Betat N, Ahrens M, Gross G 1999 Apolipoprotein E (ApoE), a Bmp-2 (bone morphogenetic protein) upregulated gene in mesenchymal progenitors (C3H10T1/2), is highly expressed in murine embryonic development. Biofactors **9:**11–17.
10. Shiraki M, Shiraki Y, Aoki C, Hosoi T, Inoue S, Kaneki M, Ouchi Y 1997 Association of bone mineral density with apolipoprotein E phenotype. J Bone Miner Res **12:**1438–1445.
11. Cauley JA, Zmuda JM, Yaffe K, Kuller LH, Ferrell RE, Wisniewski SR, Cummings SR 1999 Apolipoprotein E polymorphism: A new genetic marker of hip fracture risk—The Study of Osteoporotic Fractures. J Bone Miner Res **14:**1175–1181.
12. Johnston JM, Cauley JA, Ganguli M 1999 APOE 4 and hip fracture risk in a community-based study of older adults. J Am Geriatr Soc **47:**1342–1345.
13. Salamone LM, Cauley JA, Zmuda J, Pasagian-Macaulay A, Epstein RS, Ferrell RE, Black DM, Kuller LH 2000 Apolipoprotein E gene polymorphism and bone loss: Estrogen status modifies the influence of apolipoprotein E on bone loss. J Bone Miner Res **15:**308–314.
14. Pluijm SM, Dik MG, Jonker C, Deeg DJ, van Kamp GJ, Lips P 2002 Effects of gender and age on the association of apolipoprotein E epsilon4 with bone mineral density, bone turnover and the risk of fractures in older people. Osteoporos Int **13:**701–709.
15. Stulc T, Ceska R, Horinek A, Stepan J 2000 Bone mineral density in patients with apolipoprotein E type 2/2 and 4/4 genotype. Physiol Res **49:**435–439.
16. Booth SL, Tucker KL, Chen H, Hannan MT, Gagnon DR, Cupples LA, Wilson PW, Ordovas J, Schaefer EJ, Dawson-Hughes B, Kiel DP 2000 Dietary vitamin K intakes are associated with hip fracture but not with bone mineral density in elderly men and women. Am J Clin Nutr **71:**1201–1208.
17. Schoofs MW, van der Klift M, Hofman A, van Duijn CM, Stricker BH, Pols HA, Uitterlinden AG 2004 ApoE gene polymorphisms, BMD, and fracture risk in elderly men and women: The Rotterdam study. J Bone Miner Res **19:**1490–1496.
18. Beavan SR, Prentice A, Stirling DM, Dibba B, Yan L, Harrington DJ, Shearer MJ 2005 Ethnic differences in osteocalcin gamma-carboxylation, plasma phylloquinone (vitamin K1) and apolipoprotein E genotype. Eur J Clin Nutr **59:**72–81.
19. Zhang SH, Reddick RL, Burkey B, Maeda N 1994 Diet-induced atherosclerosis in mice heterozygous and homozygous for apolipoprotein E gene disruption. J Clin Invest **94:**937–945.
20. Kimura Y, Yanagimachi R 1995 Intracytoplasmic sperm injection in the mouse. Biol Reprod **52:**709–720.
21. Ruegsegger P, Koller B, Muller R 1996 A microtomographic system for the nondestructive evaluation of bone architecture. Calcif Tissue Int **58:**24–29.
22. Parfitt AM, Mathews CHE, Villanueva AR, Kleerekoper M, Frame B, Rao DS 1983 Relationships between surface, volume, and thickness of iliac trabecular bone in aging and in osteoporosis. Implications for the microanatomic and cellular mechanisms of bone loss. J Clin Invest **72:**1396–1409.
23. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR 1987 Bone histomorphometry: Standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res **2:**595–610.
24. Murakami H, Nakamura T, Tsurukami H, Abe M, Barbier A, Suzuki K 1994 Effects of tiludronate on bone mass, structure, and turnover at the epiphyseal, primary, and secondary spongiosa in the proximal tibia of growing rats after sciatic neurectomy. J Bone Miner Res **9:**1355–1364.
25. Gavrieli Y, Sherman Y, Ben-Sasson SA 1992 Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol **119:**493–501.
26. Sakai A, Sakata T, Tanaka S, Okazaki R, Kunugita N, Norimura T, Nakamura T 2002 Disruption of the p53 gene results in preserved trabecular bone mass and bone formation after mechanical unloading. J Bone Miner Res **17:**119–127.
27. Tanaka S, Sakai A, Tanaka M, Otomo H, Okimoto N, Sakata

T, Nakamura T 2004 Skeletal unloading alleviates the anabolic action of intermittent PTH (1-34) in mouse tibia in association with inhibition of PTH-induced increase in c-fos mRNA in bone marrow cells. J Bone Miner Res **19:**1813–1820.
28. Bakker A, Klein-Nulend J 2003 Osteoblast isolation from murine calvariae and long bones. Methods Mol Med **80:**19–28.
29. Salem GJ, Zernicke RF, Barnard RJ 1992 Diet-related changes in mechanical properties of rat vertebrae. Am J Physiol **262:**R318–R321.
30. Zernicke RF, Salem GJ, Barnard RJ, Schramm E 1995 Long-term, high-fat-sucrose diet alters rat femoral neck and vertebral morphology, bone mineral content, and mechanical properties. Bone **16:**25–31.
31. Wohl GR, Loehrke L, Watkins BA, Zernicke RF 1998 Effects of high-fat diet on mature bone mineral content, structure, and mechanical properties. Calcif Tissue Int **63:**74–79.
32. Atteh JO, Leeson S, Julian RJ 1983 Effects of dietary levels and types of fat on performance and mineral metabolism of broiler chicks. Poult Sci **62:**2403–2411.
33. Atteh JO, Leeson S 1984 Effects of dietary saturated or unsaturated fatty acids and calcium levels on performance and mineral metabolism of broiler chicks. Poult Sci **63:**2252–2260.
34. Tintut Y, Morony S, Demer LL 2004 Hyperlipidemia promotes osteoclastic potential of bone marrow cells ex vivo. Arterioscler Thromb Vasc Biol **24:**e6–10.
35. Juckett MB, Balla J, Balla G, Jessurun J, Jacob HS, Vercellotti GM 1995 Ferritin protects endothelial cells from oxidized low density lipoprotein in vitro. Am J Pathol **147:**782–789.
36. Dimmeler S, Haendeler J, Galle J, Zeiher AM 1997 Oxidized low-density lipoprotein induces apoptosis of human endothelial cells by activation of CPP32-like proteases. A mechanistic clue to the 'response to injury' hypothesis. Circulation **95:**1760–1763.
37. Bennett MR, Evan GI, Schwartz SM 1995 Apoptosis of human vascular smooth muscle cells derived from normal vessels and coronary atherosclerotic plaques. J Clin Invest **95:**2266–2274.
38. Siow RC, Richards JP, Pedley KC, Leake DS, Mann GE 1999 Vitamin C protects human vascular smooth muscle cells against apoptosis induced by moderately oxidized LDL containing high levels of lipid hydroperoxides. Arterioscler Thromb Vasc Biol **19:**2387–2394.
39. Klein BY, Rojansky N, Ben-Yehuda A, Abou-Atta I, Abedat S, Friedman G 2003 Cell death in cultured human Saos2 osteoblasts exposed to low-density lipoprotein. J Cell Biochem **90:**42–58.
40. Dietrich H, Hu Y, Zou Y, Huemer U, Metzler B, Li C, Mayr M, Xu Q 2000 Rapid development of vein graft atheroma in ApoE-deficient mice. Am J Pathol **157:**659–669.
41. Kitagawa K, Matsumoto M, Kuwabara K, Takasawa K, Tanaka S, Sasaki T, Matsushita K, Ohtsuki T, Yanagihara T, Hori M 2002 Protective effect of apolipoprotein E against ischemic neuronal injury is mediated through antioxidant action. J Neurosci Res **68:**226–232.
42. Armour KJ, Armour KE, van't Hof RJ, Reid DM, Wei XQ, Liew FY, Ralston SH 2001 Activation of the inducible nitric oxide synthase pathway contributes to inflammation-induced osteoporosis by suppressing bone formation and causing osteoblast apoptosis. Arthritis Rheum **44:**2790–2796.
43. O'Brien CA, Jia D, Plotkin LI, Bellido T, Powers CC, Stewart SA, Manolagas SC, Weinstein RS 2004 Glucocorticoids act directly on osteoblasts and osteocytes to induce their apoptosis and reduce bone formation and strength. Endocrinology **145:**1835–1841.
44. Aguirre JI, Plotkin LI, Stewart SA, Weinstein RS, Parfitt AM, Manolagas SC, Bellido T 2006 Osteocyte apoptosis is induced by weightlessness in mice and precedes osteoclast recruitment and bone loss. J Bone Miner Res **21:**605–615.
45. Chen RM, Chen TL, Chiu WT, Chang CC 2005 Molecular mechanism of nitric oxide-induced osteoblast apoptosis. J Orthop Res **23:**462–468.
46. Kim DJ, Koh JM, Lee O, Kim NJ, Lee YS, Kim YS, Park JY, Lee KU, Kim GS 2006 Homocysteine enhances apoptosis in human bone marrow stromal cells. Bone **39:**582–590.

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