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Rapid Communication

Hyperlipidemia Promotes Osteoclastic Potential of Bone Marrow Cells Ex Vivo

Yin Tintut, Sean Morony, Linda L. Demer

Objectives—Osteoporosis is associated epidemiologically with atherosclerosis and hyperlipidemia. We previously found that atherogenic lipids regulate bone formation. To determine whether hyperlipidemia also affects bone resorption, we compared osteoclastogenesis in marrow preosteoclasts derived from hyperlipidemic versus control mice.

Methods—Nonadherent marrow cells from low-density lipoprotein receptor^{-/-} (LDLR^{-/-}) and C57BL/6J mice were cultured with M-CSF and ligand for receptor activator of nuclear factor-kappaB (RANKL). Functional osteoclastic activity, measured as number of resorption pits, was significantly greater in 12-month-old LDLR^{-/-}. Similar results were obtained in 5- and 10-month-old LDLR^{-/-} versus C57BL/6J mice on a high-fat diet. Osteoclastic differentiation, indicated by tartrate resistant acid phosphatase (TRAP) activity, was significantly greater in the 12-month-old LDLR^{-/-}, and there was a trend toward increased TRAP activity in LDLR^{-/-} on a high-fat diet, at ages 5 and 10 months. Osteoclastic parameters correlated with total serum lipoproteins with a possible threshold effect. Osteoporotic human cortical bone stained positive for lipids in the perivascular space of Haversian canals by oil red O. The presence of lipid hydroperoxides was detected in bone marrow from hyperlipidemic mice.

Conclusions—Hyperlipidemia may contribute to osteoporosis via increased osteoclastic bone resorption. (*Arterioscler Thromb Vasc Biol.* 2004;24:e6–e10.)

Key Words: calcification ■ osteoclast ■ atherosclerosis ■ hyperlipidemia ■ osteoporosis

Epidemiological studies link osteoporosis with hyperlipidemia and atherosclerosis.¹ In some populations, this relationship is independent of age.² Lipid-lowering treatments reduce osteoporosis³ in some, but not all, studies. In mice, atherosclerosis susceptibility corresponds with low bone density.⁴

Osteoclasts are formed by fusion of mononuclear cells of the monocyte-macrophage lineage into multinucleate polar cells that form resorption lacunae. Until recently, in vitro osteoclastogenesis required co-culture with marrow stromal cells. It is now known that two growth factors substitute for stromal cells: monocyte colony-stimulating factor (M-CSF) and the ligand for receptor activator of nuclear factor-kappaB (RANKL).⁵ M-CSF, which is well known for its role in foam cell formation, is a key growth factor for osteoclasts that is required for survival and fusion of preosteoclasts into the mature multinucleated form.⁶ Cells committed to the osteoclast lineage contain high levels of tartrate-resistant acid phosphatase (TRAP) activity. Functional osteoclastic activity is evidenced by formation of resorption pits in hydroxyapatite mineral.

We previously showed that hyperlipidemia reduces bone density in vivo⁷ and that this may be explained in part by inhibition of osteoblastic (bone-forming cell) differentiation by bioactive lipids.⁸ However, osteoporosis results primarily from excess bone resorption rather than inadequate bone formation. In vitro, atherogenic lipids promote osteoclastic differentiation,⁹ but the in vivo effects are not known. We assessed osteoclastic differentiation and resorptive activity in marrow preosteoclasts derived from mice with genetic and diet-induced hyperlipidemia and from controls. Results showed that osteoclastic activity was greater in hyperlipidemic compared with control mice and that lipid deposits were present in the perivascular space of Haversian canals in osteoporotic bone.

Methods

C57BL/6J and low-density lipoprotein receptor^{-/-} (LDLR^{-/-}) mice on a C57BL/6J background were obtained from the Jackson Laboratory (Bar Harbor, ME). Chow diet (7013) and high-fat diet (TD 94059, a standard atherogenic diet without cholate) were

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Serum Lipoprotein and Subfraction Levels (in mg/dL) and Osteoclastic Activity Parameters for LDLR^{-/-} and Control C57BL/6J Mice on High-Fat and Chow Diets

Mouse Strain	Diet	N	Total Cholesterol	LDL	TRAP-Positive Cells	Resorption Pits
LDLR ^{-/-}	High-fat	4	1230±200	1131±205	558±134(a)	189±19 (c)
	Chow	4	295±48	205±39	482±40 (b)	169±59(d)
C57BL/6J	High-fat	2	139±NA	18.4±NA	392±NA	108±NA
	Chow	4	84±10	13.2±1	326±24 (a,b)	106±6 (c,d)

(a) $P<0.005$ for LDLR^{-/-} on high-fat vs C57BL/6 on chow.

(b) $P=0.06$ for LDLR^{-/-} on chow vs C57BL/6 on chow.

(c) $P<0.01$ for LDLR^{-/-} on high-fat vs C57BL/6 on chow.

(d) $P=0.07$ for LDLR^{-/-} on chow vs C57BL/6 on chow.

N indicates number of mice; NA, not applicable.

obtained from Harlan-Teklad (Madison, WI). Lipoprotein levels were determined as described previously.¹⁰

Preosteoclast Cell Culture

Whole bone marrow was isolated from long bones and plated overnight in 100-mm plates with 5 ng/mL M-CSF. The next day, nonadherent cells were removed and were plated at 3×10^5 cells per well in 96-well plates or on osteologic discs (BDPharMingen, San Diego, CA) in alpha-MEM (Irvine Scientific) supplemented with 10% FBS (Hyclone; Logan, UT), sodium pyruvate (1 mmol/L), and penicillin and streptomycin (10 U/mL). Mouse recombinant M-CSF (25 ng/mL; R&D Systems; Minneapolis, MI) and mouse recombinant RANKL (40 ng/mL; R&D systems; Minneapolis, MI) were added at that time and on day 3 when media was replenished. Assays were performed on days 5 through 7.

TRAP Assay

TRAP activity was assayed using the TRAP cytochemical stain technique as described previously⁹ for all groups, except the 7-month-old mice for which TRAP activity was assayed in whole-cell extract using the TRAP colorimetric activity assay, also described previously.⁹

Resorption Pit Assay

After 6 to 7 days, the cells were removed from the discs and the resorption pits were counted in quadruplicate wells by a blinded observer. Results are expressed as average number of resorption pits per osteologic disc more than 4 discs.

Histomorphometry

Femurs were fixed in 95% ethanol, embedded in plastic, cut into 6-micron longitudinal sections, and stained with the Goldner trichrome method. One bone from the LDLR^{-/-} group was excluded because of technical difficulties during histological preparation. Osteoclasts were identified as red-orange stained cells found associated with bone or cartilage. Osteoclasts, within 1 to 2 cell diameters of the distal epiphyseal growth plate, were counted by an experienced observer blinded to the subject group and normalized to the curvilinear growth plate length using image analysis software (Sigma Scan Pro v.5.; SPSS, Chicago, IL). Cell size was indexed as the mean number of nuclei per osteoclast.

Oil Red O Stain

Fresh specimens of human nonosteoporotic and osteoporotic femoral head bones were obtained from surgical patients by approval of the Institutional Review Board. Bones were decalcified in 10% EDTA until soft enough to be cut by microtome. The tissues were embedded in OCT, cyrosectioned, and stained with oil red O.

Lipid Hydroperoxide Assay

Four C57BL/6 mice were fed a high-fat diet for 5 weeks, and 3 were fed a high-fat diet for 16 weeks. Bone marrow was harvested from

the femurs, and lipids were extracted with chloroform/methanol and assayed for lipid hydroperoxides as previously described.¹¹

Statistical Analysis

Values are expressed as mean±SD. Data shown in Table 1 were analyzed by ANOVA with Tukey post-hoc analysis. Significance level was set at $P<0.05$. Data shown in the figure were analyzed using Spearman correlation analysis, a rank-based method.¹²

Results

At age 5 months, marrow from LDLR^{-/-} mice receiving a high-fat diet for 2 months had increased osteoclastic potential compared with that from C57BL/6J mice also receiving a high-fat diet for 2 months. There was a nonsignificant trend toward increased TRAP-positive cells in the LDLR^{-/-} mice (601 ± 248 versus 292 ± 189 ; $n=4$ per group; $P<0.1$). Resorption pit formation was significantly greater in cells from LDLR^{-/-} mice (254 ± 120 versus 27 ± 12 pits per osteologic disc; $n=4$ per group; $P<0.01$).

To determine whether this relationship remained with older mice and with a quantitative TRAP assay, marrow was obtained from 10-month-old LDLR^{-/-} and C57BL/6J mice receiving a high-fat diet for 7 months. Marrow cells were assayed for quantitative TRAP activity in addition to the resorption pit formation assay. In this group, there was also a trend toward increased TRAP activity in the marrow of LDLR^{-/-} mice (2.6 ± 1.5 versus 1.7 ± 0.8 U; $n=5$ per group; $P=0.24$). The marrow of LDLR^{-/-} mice also had greater mineral resorptive activity than the fat-fed C57BL/6J mice (213 ± 83 versus 6 ± 2 pits per osteologic disc; $n=5$ per group; $P<0.05$). The results from these two age groups suggest that hyperlipidemia leads to an increase in osteoclastic function and a nonsignificant increase in osteoclastic differentiation.

To further assess the relationship between diet-induced and genetically induced hyperlipidemia and osteoclastic potential, we compared 4 groups of 5-month-old mice: C57BL/6J mice on a chow ($n=4$) or high-fat ($n=2$) diet and LDLR^{-/-} mice on a chow ($n=4$) or high-fat ($n=4$) diet for 2.5 months. Initially, 4 C57BL/6 mice were in the high-fat group, but 2 had to be excluded because of a diet error. There were significant differences in serum lipid levels and in osteoclastic resorptive activity and TRAP positivity (Table 1). To determine whether their osteoclastic potential correlated with severity of hyperlipidemia, we used rank-based Spearman correlation analysis comparing these same measures of oste-

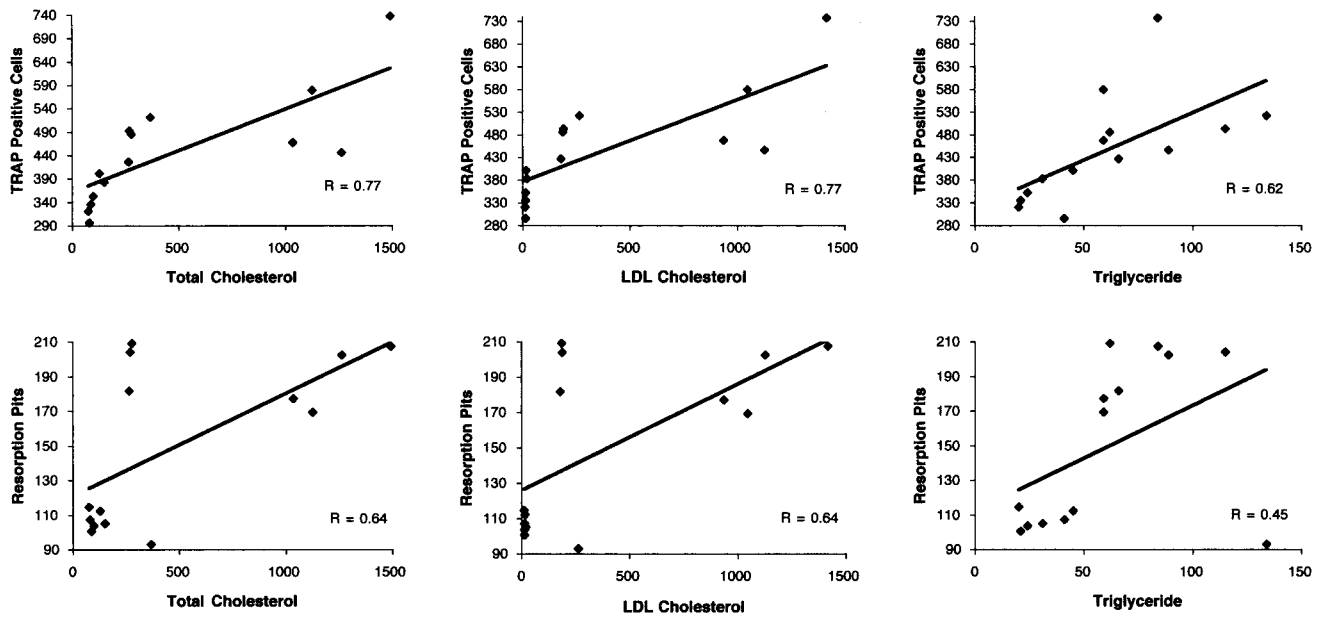


Figure 1. Correlation of osteoclastic potential (TRAP-positive cells and resorption pits) versus lipid levels (total cholesterol, LDL cholesterol, and triglyceride) in marrow preosteoclasts from 5-month-old LDLR^{-/-} and C57BL/6J mice on chow and high-fat diets. (R_s =Spearman correlation coefficient) The low R value for the resorption pits versus triglyceride may be because of the outlier (bottom right panel).

osteoclastic activity with serum lipoproteins. TRAP positivity correlated with total and LDL cholesterol and triglycerides, and similar relationships were found for resorption pit formation (Figure 1).

To determine whether older mice also have a greater effect on osteoclast parameters, marrow from LDLR^{-/-} and C57BL/6J mice at age 12 months was isolated and cultured as described, and blood lipid levels were determined. Nonad-

herent marrow cells from the chow-fed LDLR^{-/-} mice had significantly more TRAP-positive cells (760 ± 128 versus 280 ± 132 ; $n=6$ per group; $P<0.001$) and significantly greater mineral resorptive activity (110 ± 59 versus 19 ± 7 pits per osteologic disc; $n=6$ per group; $P<0.01$) than marrow cells from the chow-fed C57BL/6J mice of the same age. TRAP-positive cells and resorptive activity correlated with total and LDL cholesterol by Spearman analysis ($P<0.01$ for

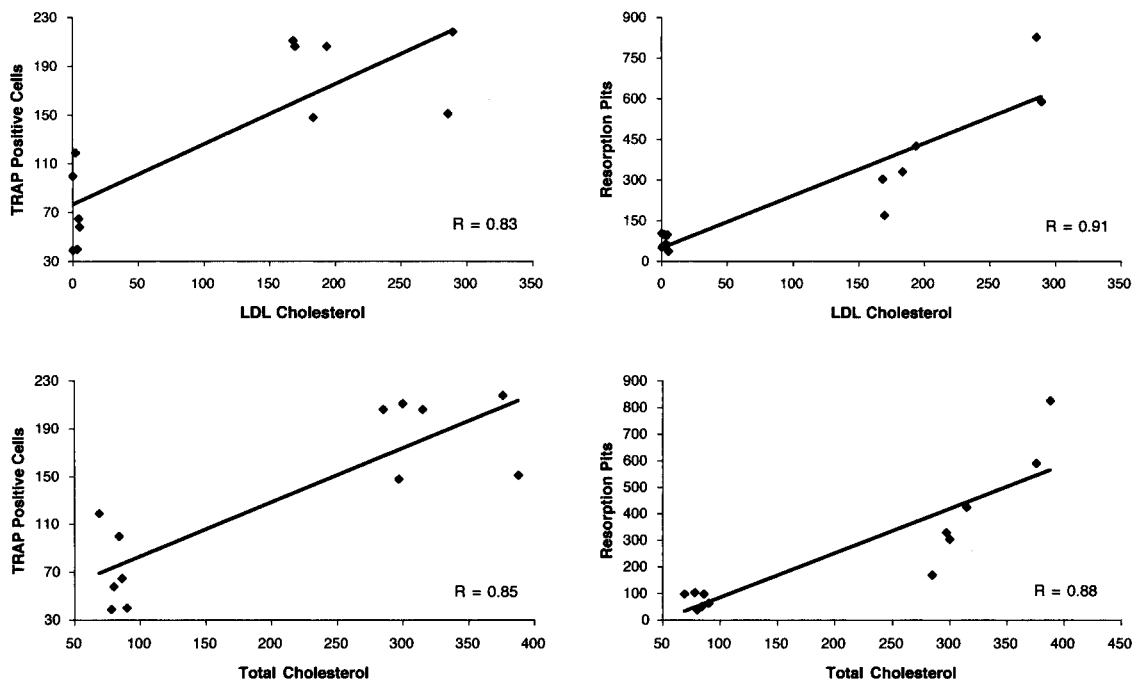


Figure 2. Correlation of osteoclastic potential versus lipid levels in marrow preosteoclasts from 12-month-old LDLR^{-/-} and C57BL/6J chow-fed mice. (R_s =Spearman correlation coefficient)

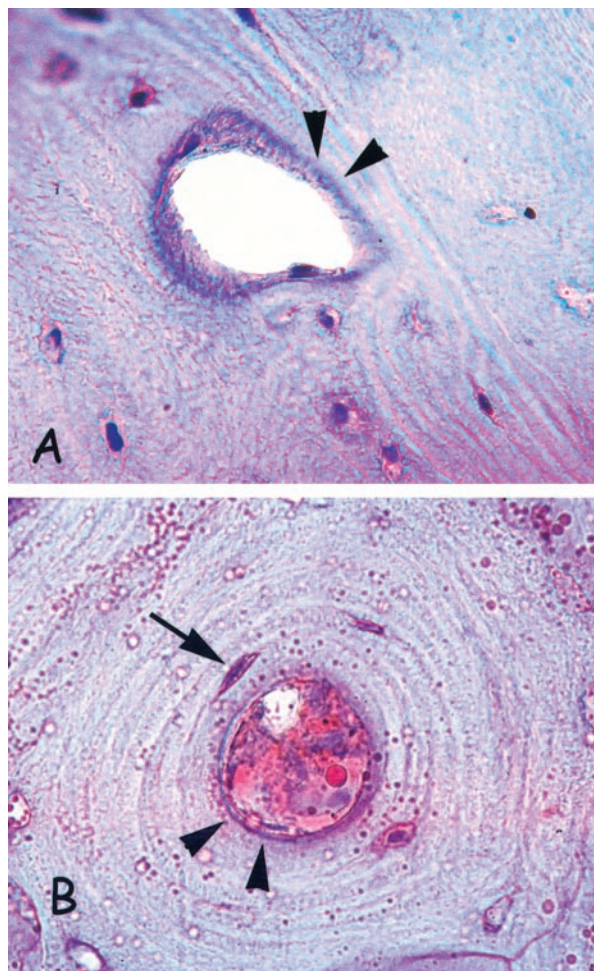


Figure 3. Oil red O stain (red) of human cortical bones from nonosteoporotic (A) or osteoporotic (B) patients. → indicates typical osteocyte; ►, perivascular space of the Haversian canal, which has a diameter of $\approx 40 \mu\text{m}$.

each; Figure 2). These parameters also correlated positively with serum triglyceride and free fatty acid levels (data not shown).

To examine the osteoclast size and numbers in these mice, histomorphometry was performed on their bones. Results from 7 femurs (4 C57BL/6J and 3 LDLR $^{-/-}$) showed 7.5 ± 1.2 osteoclasts per millimeter of plate length in the control (C57BL/6) bones and 7.7 ± 2.1 osteoclasts per millimeter of plate length for femurs from the hyperlipidemic mice, with no significant difference ($P=0.91$). However, the mean osteoclast size index was greater in the bones of the hyperlipidemic LDLR $^{-/-}$ mice (3.2 ± 0.3 versus 2.6 ± 0.2 nuclei/osteoclast; $P<0.04$).

We tested for the presence of oxidized lipids in bone by measuring lipid hydroperoxides in bone marrow from mice fed a high-fat diet for 5 weeks. Results showed a mean of 605.8 ± 32.5 ng of lipid hydroperoxide per bone. To determine whether duration of diet influenced the deposition of lipids, we performed an additional experiment in which 3 C57BL/6 mice were fed a high-fat diet for 16 weeks and the marrow was assayed as previously described. Results showed a mean of 3699 ± 1677 ng of lipid hydroperoxides per bone.

To assess whether lipids deposit within the perivascular space of Haversian canals in human bone, we obtained fresh specimens of human osteoporotic and nonosteoporotic femoral head bone from surgical patients. Cryosections stained with oil red O showed positive staining for lipid within the perivascular space of Haversian canals of cortical bone in the osteoporotic patient (Figure 3). Lipid deposits were also faintly detected in the osteocytes (Figure 3).

Discussion

Osteoporosis has been linked with atherosclerosis and hyperlipidemia. Our results show that osteoclastic potential is greater in preosteoclasts from bone marrow in hyperlipidemic compared with normal mice. The increased osteoclast size in bones of hyperlipidemic mice suggests a functional, but not numeric, difference in osteoclasts. Evidence of lipid oxidation products was also found in bone marrow of hyperlipidemic mice, further suggesting that hyperlipidemia promotes bone resorptive potential in vivo.

Cortical and trabecular bone are vascularized, and bone cells are exposed to lipoproteins from the circulation. Our findings indicate that lipids are present in the perivascular space of the Haversian canals in osteoporotic cortical bone. The faintly positive staining in osteocytes may be related to the need for the fat-soluble vitamins D and K in bone metabolism.¹³ In hyperlipidemic conditions, the lipoproteins may accumulate and undergo oxidation in the same sub-endothelial perivascular space of bone remodeling units as they do in arteries.

In the present study, the degree of hyperlipidemia correlated with the degree of osteoclastic functional activity in the marrow and correlated less well with TRAP staining and quantitative activity in the younger mice. This may be caused by either the number of subjects being too few to reach significance or the lipids acting primarily on osteoclastic enzymes other than TRAP. The variation in baseline values for resorption pits between experiments may be caused by differences in reagent lots, assay techniques, or inter-animal differences.

The correlation between lipid levels and parameters of osteoclast activity were assessed using Spearman correlation analysis, a method based on ranking of the values of the 2 parameters. We also tested linear, exponential, and logarithmic models for closeness of fit to the data. All 3 models provided good correlations, but no single model was consistently the best. Therefore, we chose the nonparametric, rank-based method for all the correlations to allow comparison without having to assume normal distributions for the parameters. Interestingly, a threshold effect at 250 to 300 mg/dL cholesterol is suggested (Figure 2). In C57BL/6 mice, the diet had little effect on cholesterol level, and the osteoclast parameters were not significantly affected. In the LDL $^{-/-}$ mice, the diet did increase cholesterol level significantly but still had little effect on osteoclast parameters, further supporting the threshold effect (Table 1).

Previously, we showed that osteoblastic differentiation is inhibited by modified lipoproteins and lipids⁸ and that bone density is reduced in hyperlipidemic mice in vivo.⁷ We also found that marrow stromal cells from mice fed the high-fat

diet have reduced osteoblastic potential,¹⁴ consistent with the findings of Verma et al that fat tissue replaces marrow hematopoietic tissue in osteoporotic bone.¹⁵ Recently, we reported that lipid oxidation products enhance the osteoclastic differentiation of bone marrow preosteoclasts in vitro.¹⁴ We show that osteoclastic differentiation is enhanced in vivo by hyperlipidemia. Such effects would explain the epidemiological findings linking hyperlipidemia and osteoporosis.

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