Isomer-Specific Effects of Conjugated Linoleic Acid on Mineralized Bone Nodule Formation from Human Osteoblast-Like Cells

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Mixed isomers of conjugated linoleic acid (CLA) have been shown to have variable effects on bone formation and resorption in animals. The variable effects of CLA on bone physiology may be due to the different isomers present in common commercial preparations of CLA, and the effects of the predominant individual isomers (9*cis*,11*trans* and 10*trans*,12*cis* CLA) are not clear. The objective of this study was to determine the effects of individual and mixed isomers of CLA on mineralized bone nodule formation and alkaline phosphatase (ALP) activity *in vitro* using long-term cultures of SaOS-2 cells. Mineralized bone nodules were stained using the von Kossa method, and ALP activity in cell lysates was measured as a marker of early osteoblast differentiation. The 9*cis*,11*trans* isomer increased the number (~4- to 11-fold) and size (~2- to 5-fold) of mineralized bone nodules from 25 to 100 μM, but the 10*trans*,12*cis* isomer did not. The increase in mineralized bone nodule formation by 9*cis*,11*trans* CLA was accompanied by a variable increase in ALP activity. These results show that the 9*cis*,11*trans* isomer of CLA increases the formation of mineralized bone nodules using bone cells of human origin, and provide evidence for isomer-specific effects of CLA on bone health. Exp Biol Med 232:246–252, 2007

Key words: conjugated linoleic acid; osteoporosis; SaOS-2 cells; bone; alkaline phosphatase

Introduction

Osteoporosis is a major cause of morbidity and mortality; it is characterized by decreased bone mass, increased bone fragility, and increased susceptibility to bone fractures (1). Attaining maximal peak bone mass through proper nutrition and lifestyle choices is an important strategy for preventing osteoporosis later in life (2, 3). Consumption of dairy products is associated with improved bone health in a variety of different populations (4). Although calcium and vitamin D are major nutrients in dairy products that promote bone health, other constituents, such as conjugated linoleic acid (CLA), may also be important (5–14). CLA refers to a group of positional and geometric isomers of linoleic acid that are produced by the bacterial biohydrogenation of linoleic acid *via* an enzymatic isomerase reaction (15). CLA has diverse physiological effects (16, 17) and is found naturally in foods from ruminant animals, predominantly as the 9*cis*,11*trans* isomer (18). Synthetic CLA preparations are abundant in both the 9*cis*,11*trans* and 10*trans*,12*cis* isomers. These isomers have both been shown to be biologically active (19) and are known to have different physiological effects. Several studies have examined the effects of CLA on the development of chronic diseases such as cancer, diabetes, and cardiovascular disease (20–24), and there is growing evidence that CLA may also affect bone health (5–14, 25–32).

Several studies using experimental animals have shown equivocal effects of CLA on bone formation (5–14, 25, 26, 31, 32). Rodent studies have shown that dietary CLA supplementation increases body ash (10, 11, 31, 32), suggesting a favorable effect of CLA on bone formation. However, other animal studies showed no effect of CLA on bone mineral content (7, 25). CLA has also been shown to increase (8, 9) or decrease bone formation rates (12) and to have variable effects on markers of bone formation and resorption (13, 14). In rodent calvarial cells, CLA alters protein levels of the osteoblast-specific transcription factor core binding factor alpha 1 (Cbfa1) and increases osteoblast differentiation (26).

Only a few studies have examined the effects of CLA on human bone physiology (27–30). Intake of dietary CLA has been shown to be positively associated with bone

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mineral density in postmenopausal women (27), while supplemental CLA has been shown to have no effect on bone mineral density in men (28, 29). Using bone cells of human origin, individual and mixed isomers of CLA have been recently shown to have variable stimulatory effects on alkaline phosphatase (ALP) activity (30), a marker of osteoblast differentiation. Osteoblasts are mononucleated bone-forming cells that actively synthesize and secrete an organic bone matrix which undergoes rapid mineralization (33). ALP regulates bone matrix mineralization by hydrolyzing phosphate esters to increase the local phosphate concentration necessary for bone formation (34, 35). Although CLA has been shown to variably increase ALP activity, the effects of individual and mixed CLA isomers on mineralized bone nodule formation using bone cells of human origin are not known. The objective of the present study is to determine the direct effects of the individual and mixed isomers of CLA on osteoblastic bone formation and differentiation using the human osteoblast-like SaOS-2 cell line.

Materials and Methods

Materials. SaOS-2 cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). The 9*cis*,11*trans* and 10*trans*,12*cis* (>98% pure) isomers of CLA were purchased from Matreya (Pleasant Gap, PA). Mixed CLA isomers (~41% 9*cis*,11*trans*/9*trans*,11*cis*; ~44% 10*trans*,12*cis*; ~10% 10*cis*,12*trans*; ~5% 9*trans*,11*trans*, 10*trans*,12*trans* and 9*cis*,11*cis*) were purchased from Nu-Chek Prep (Elysian, MN). The Bio-Rad Protein Assay reagent was purchased from Bio-Rad (Mississauga, Canada). Fetal bovine serum (FBS) was purchased from Cansera (Etobicoke, Canada). Antibiotic-antimycotic was purchased from GIBCO (Burlington, Canada). Ham's F12 and PBS were purchased from Central Technical Services, University of Toronto. Alkaline phosphatase assay reagent was purchased from Teco Diagnostics (Anaheim, CA). β-Glycerophosphate was purchased from ICN Pharmaceuticals (Costa Mesa, CA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. Cells were grown in 24-well dishes at a density of 5×10^3 cells per well in Ham's F12 medium, containing 10% FBS, 28 m*M* HEPES buffer (pH 7.35), 1.4 m*M* $CaCl_2$, 2 m*M* glutamine, 1% antibiotic-antimycotic solution, and 50 μg/ml ascorbic acid. The medium was changed every 2–3 days. On the eighth day and at every medium change thereafter, the medium was replaced with medium containing 10 m*M* β-glycerophosphate as well as varying concentrations (25, 50, and 100 μ*M*) of mixed, 9*cis*,11*trans,* or 10*trans*,12*cis* CLA or vehicle (0.1% ethanol). CLA was added to the medium by first dissolving it in ethanol, which was then added to FBS supplemented with 1 g/l of fatty acid–free bovine serum albumin (BSA). The final concentration of ethanol and BSA in each well was 0.1% and 1 g/l, respectively.

Determination and Quantification of Mineralized Bone Nodules. After 21 days in culture, the cells were washed twice with PBS, fixed overnight with 4% *p*-formaldehyde, and stained *in situ* using the standard von Kossa technique (36, 37). Briefly, the nodules were stained with 5% silver nitrate under UV light for 30 mins, background color was removed with 5% sodium thiosulfate, and the cells were maintained in 50% glycerol. The mineralized nodule areas and numbers were quantified using a FluorChem imaging system.

Alkaline Phosphatase (ALP) Activity. After 2, 4, and 10 days of treatment, the cells were washed twice with 50 m*M* Tris-HCl (pH 7.35). Cells were lysed in buffer containing 0.05% Triton-X-100 in 50 m*M* Tris-HCl (pH 7.35) following one freeze-thaw cycle. ALP activity was determined according to the method of Lowry (38) in cell sonicates using a commercially available kit from Teco Diagnostics. ALP activity was determined as the amount of *p*-nitrophenol produced from *p*-nitrophenyl phosphate over time at 37°C, standardized for protein concentration. The absorbance of *p*-nitrophenol was measured at 405 nm using a Fusion plate reader every 2 mins for 15 readings. Protein concentration of the cell sonicates was determined using the Bio-Rad Protein Assay reagent. The ALP activity of each sample was normalized to its protein concentration, calculated as units/g protein, and expressed as percent of control. One unit of ALP activity is defined as the amount of enzyme that catalyzes the conversion of one micromole of *p*-nitrophenyl phosphate to *p*-nitrophenol and phosphate per minute.

Statistical Analyses. Results are expressed as mean ± SEM with at least three replicates in each group. Differences were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. The effects of each CLA treatment were assessed on separate plates, with each plate having its own controls. The effects of CLA on mineralized bone nodule formation and ALP activity were analyzed relative to the controls on the corresponding plates. P values <0.05 were considered significant. All data were analyzed using GraphPad Prism Software, Version 4.00.

Results

The Effects of CLA on the Formation of Mineralized Bone Nodules. Mineralized bone nodules formed from SaOS-2 cells were first observed after 10 days of treatment (Day 18). The nodules appeared three-dimensional in appearance under a phase contrast microscope and grew in size until the end of the culture period (Day 21). After von Kossa staining, the mineralized bone nodules could be observed with the naked eye as dark spots. Mixed and 9*cis*,11*trans* CLA increased the formation of mineralized bone nodules from these cells (Fig. 1). Mixed CLA isomers at concentrations of 50 and 100 μ*M* increased both

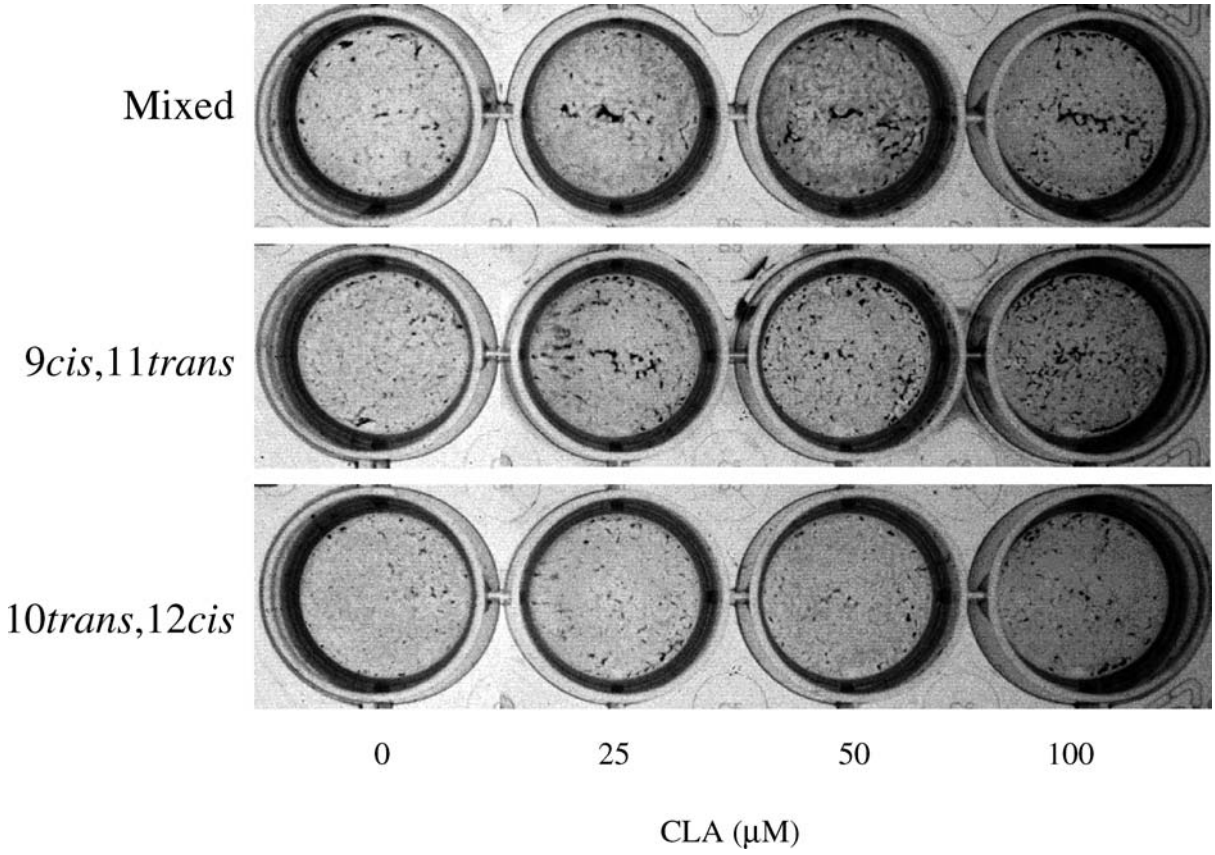

Figure 1. Effects of mixed and individual isomers of CLA on the formation of mineralized bone nodules in SaOS-2 cells cultured in 24-well plates fixed on Day 21 and stained using the von Kossa method. Cells were treated with either vehicle or mixed, 9*cis*,11*trans* or 10*trans*,12*cis* CLA at concentrations of 25, 50, and 100 μ*M*.

the number and size of mineralized bone nodules formed, as quantified using a FluorChem imaging system. Compared to control cells, 50–100 μ*M* mixed CLA increased the area of nodules by approximately 3- to 4.5-fold (Fig. 2A), while 100 μ*M* increased the number of nodules by approximately 5-fold (Fig. 2B). The 9*cis*,11*trans* isomer of CLA increased both the number and size of mineralized bone nodules formed. Compared to control cells, 25–100 μ*M* 9*cis*,11*trans* CLA increased the area of nodules formed by approximately 2- to 5-fold (Fig. 2C), while 25–100 μ*M* of the 9*cis*,11*trans* isomer of CLA increased the number of nodules by approximately 4- to 11-fold (Fig. 2D). In contrast, 10*trans*,12*cis* CLA did not appear to increase the formation of mineralized bone nodules at any of the concentrations tested (Fig. 2E and F).

Effects of CLA on ALP Activity. The effect of increasing concentrations of mixed, 9*cis*,11*trans*, and 10*trans*,12*cis* CLA on ALP activity in SaOS-2 cells is shown in Figure 3. As shown in Figure 3A, 25 μ*M* mixed CLA increased ALP activity after 2 days of treatment by approximately 40%, while 50 and 100 μ*M* mixed CLA increased ALP activity after 4 days of treatment by approximately 110% and 90%, respectively. As shown in Figure 3B, 100 μ*M* 9*cis*,11*trans* CLA increased ALP activity at all time points by approximately 40%. After 4 days of treatment, 25 μ*M* increased ALP activity by approximately 40%, while 50 μ*M* increased ALP activity by approximately 100%. As shown in Figure 3C, 10*trans*,12*cis* CLA increased ALP activity at a later time in culture beginning at 4 days of treatment. At this time point, 50 μ*M* 10*trans*,12*cis* CLA increased ALP activity by approximately 50%, while 100 μ*M* increased ALP activity approximately 65%, compared to control cells. After 10 days of treatment, 100 μ*M* increased ALP activity by approximately 40%.

Discussion

In the present study we have shown that 9*cis*,11*trans* CLA, the most abundant isomer found in food products from ruminant animals, increases the formation of mineralized bone nodules from human osteoblast-like cells. This effect was accompanied by an increase in osteoblast differentiation as measured by ALP activity. Although mixed CLA isomers also increased both the formation of mineralized bone nodules and ALP activity, these effects were likely due to the 9*cis*,11*trans* isomer, because the effects were intermediate between those of the 9*cis*,11*trans* and 10*trans*,12*cis* isomers. These results suggest a potential beneficial effect of the 9*cis*,11*trans* isomer of CLA on bone

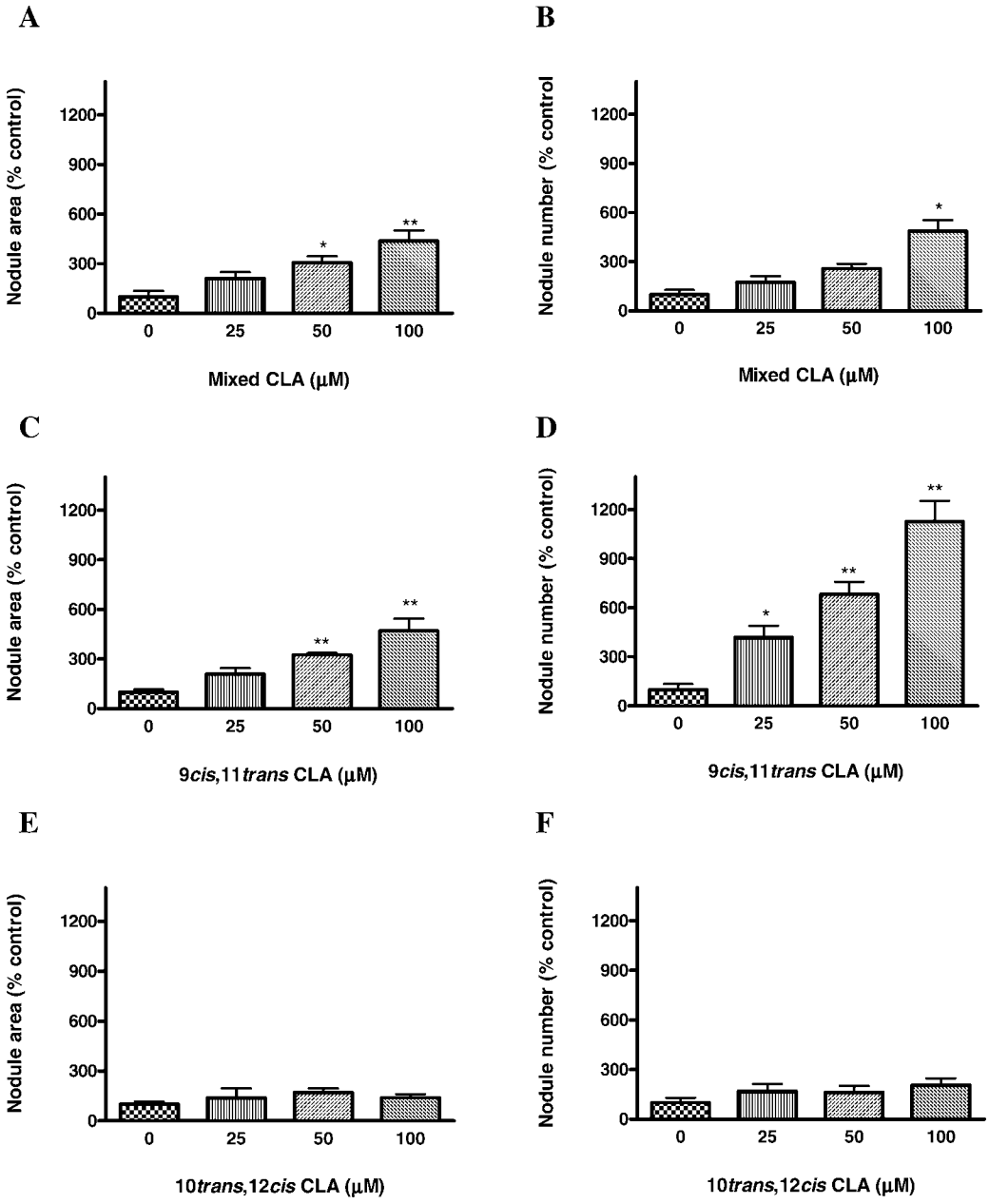

Figure 2. Effects of individual and mixed CLA isomers on the area and number of mineralized bone nodules formed. Cells were treated with either vehicle or one of mixed (A, nodule area; B, nodule number), 9*cis*,11*trans* (C, nodule area; D, nodule number), or 10*trans*,12*cis* (E, nodule area; F, nodule number) CLA at concentrations of 25, 50, and 100 μ*M*. Values are expressed as mean ± SEM. The effect of concentrations of individual fatty acids on mineralized bone nodule formation was analyzed using one-way ANOVA. **P* < 0.05, ***P* < 0.01, compared to respective control (0 μ*M*).

health in humans. To our knowledge, this is the first study to examine the effect of CLA on mineralized bone nodule formation *in vitro*, although a few studies have examined its effects on ALP activity (8, 26, 30). Our results are consistent with the findings of another study which demonstrated that individual and mixed CLA isomers variably increase ALP activity in human osteoblast-like cells (30). Similarly, CLA has also been shown to increase ALP activity in murine calvarial cells; however, this effect was observed with the 9*cis*,11*trans* but not the 10*trans*,12*cis* isomer (26). In murine calvarial cells, treatment with 9*cis*,11*trans* CLA for 7 days increased, while 14-day treatment decreased, the expression of Cbfa1 in murine calvarial cells (26). Cbfa1 expression is required for early osteoblast differentiation but negatively regulates late-stage osteoblast differentiation (39), suggesting that 9*cis*,11*trans* CLA may regulate bone formation through Cbfa1 expression. This hypothesis is consistent with our results showing that 9*cis*,11*trans* CLA increases early osteoblast differentiation as assessed by an increase in ALP activity.

There are several possible explanations for the isomer-specific effects of CLA that we observed on mineralized

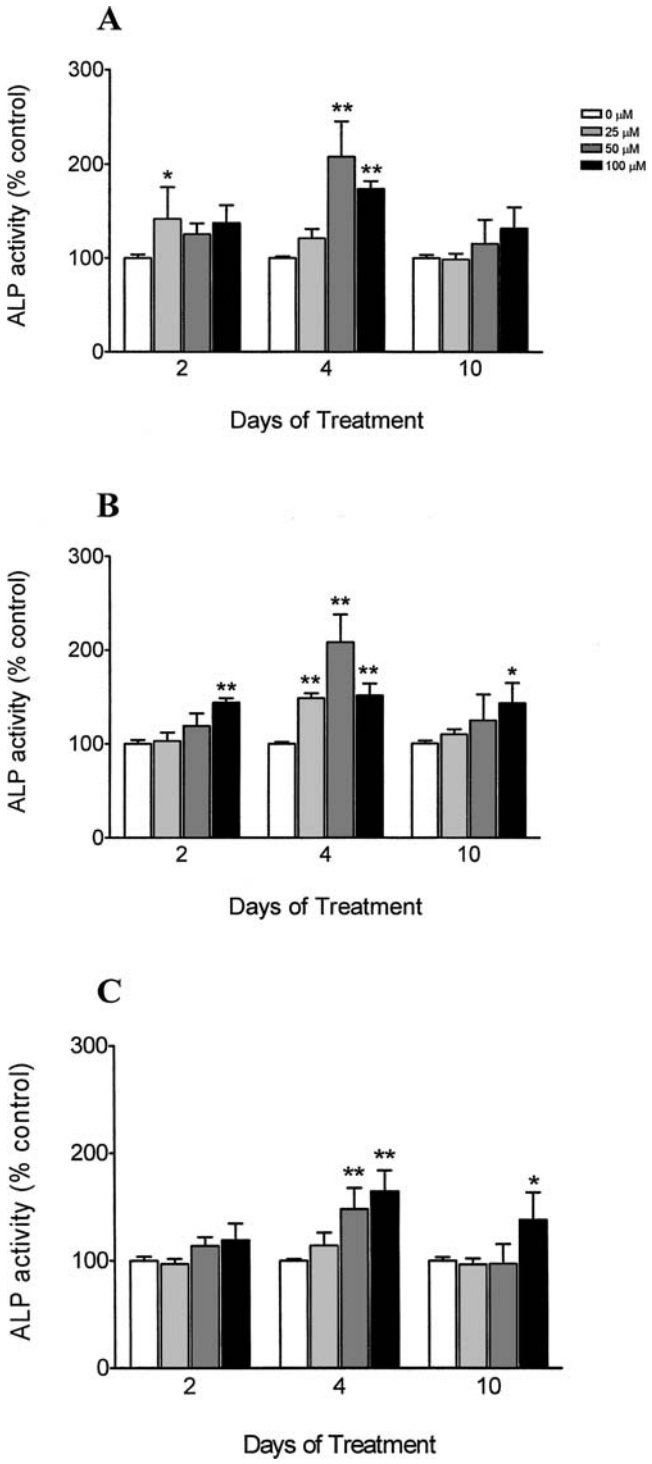

Figure 3. Effects of individual and mixed CLA isomers on ALP activity. ALP activity was measured in cells treated with either vehicle or one of mixed (A), 9*cis*,11*trans* (B), or 10*trans*,12*cis* (C) CLA isomers at concentrations of 25, 50, and 100 μM for 2, 4, or 10 days. Values are expressed as mean ± SEM. The effect of concentration of individual fatty acids on ALP activity was analyzed using one-way ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to respective control (0 μM).

bone nodule formation. One rodent study demonstrated that 9*cis*,11*trans* and 10*trans*,12*cis* CLA are preferentially retained by different organs and tissues. Compared to 10*trans*,12*cis* CLA, the 9*cis*,11*trans* isomer is preferentially retained by bone tissues, and of the tissues analyzed, bone appears to retain the highest levels of CLA (40). This suggests that 9*cis*,11*trans* CLA may affect bone formation through direct effects on bone physiology. The stimulatory effect of 9*cis*,11*trans* CLA on mineralized bone nodule formation could also result from favourable alterations in prostaglandin E_2 (PGE_2) biosynthesis in these cells. PGE_2 is a major regulator of bone metabolism (41, 42). At high concentrations (10^{-6} *M*) *in vitro* PGE_2 inhibits bone formation (41) and stimulates bone resorption (42), while at low concentrations (10^{-10}–10^{-8} *M*) PGE_2 stimulates bone formation (41). It was recently shown that relative to vehicle, mixed and 10*trans*,12*cis* CLA decrease PGE_2 biosynthesis in SaOS-2 cells and human osteoblastic MG-63 cells to a greater extent than 9*cis*,11*trans* CLA (30). Thus, 10*trans*,12*cis* CLA may reduce PGE_2 levels below that which is required for bone formation, whereas 9*cis*,11*trans* CLA may reduce PGE_2 levels to within the range that stimulates bone formation. It is also possible that the 9*cis*,11*trans* CLA increases mineralized bone nodule formation by decreasing 3-hydroxy-3-glutaryl CoA (HMG-CoA) reductase. Statins, which inhibit HMG-CoA reductase, have been shown to increase bone formation *in vitro* and *in vivo* by stimulating BMP-2 expression (43).

Several studies using experimental animals have shown equivocal effects of mixed CLA isomers on bone formation. Mice fed 5 g/kg dietary CLA had higher levels of whole-body ash (11), and chickens had higher tibial bone ash (10), suggesting that CLA may enhance bone formation. Chicks fed 5.2 g/kg butterfat, a rich source of CLA, were found to have 60% higher bone formation rates (9). Furthermore, a diet containing 5 g/kg CLA increased the tibial bone formation rate in rats (8). The studies showing a null or negative effect of dietary CLA on bone physiology provided CLA as 10 g/kg of the diet (7, 12, 13). At this concentration, CLA did not alter markers of bone formation (osteocalcin) or resorption (urinary pyridinium cross-links) in Wistar rats (13), but it reduced the tibial mineral apposition and bone formation rates of weanling Sprague-Dawley rats without affecting bone mineral content (12). In weanling rats with polycystic kidney disease, which is characterized by elevated tissue levels of arachidonic acid, elevated parathyroid hormone, and reduced bone mass, 10 g/kg CLA had no effect on bone mineral density (7).

All of the above studies were conducted using young, growing male animals. Although there may be important sex differences in lipid metabolism and bone physiology, few studies have examined the effect of CLA on bone in aging female animals. Because the rate of bone loss is accelerated after menopause, ovariectomized animals are useful models for studying the effect of CLA on bone resorption. For example, ovariectomized Fisher rats fed 5–

10 g/kg CLA displayed less bone resorption compared to control rats (14). In nonovariectomized female pigs, however, 0.7–5.5 g/kg dietary CLA did not alter bone mineral content (25). Inconsistencies between animal studies may be due to a number of differences between studies, including the sex, species, or strain of the animal model used or differences in the dose or duration of CLA treatment. In addition, commercially prepared diets may contain varying concentrations of 9*cis*,11*trans* and10-*trans*,12*cis*, as well as other isomers of CLA.

To our knowledge, the present study is the first to examine the direct effects of CLA on mineralized bone nodule formation using bone cells of human origin. We demonstrate that 9*cis*,11*trans* CLA greatly increases the formation of mineralized bone nodules in long-term cultures of human SaOS-2 cells. Because bone formation is tightly coupled with resorption in healthy bone, ongoing studies aim to determine whether CLA affects bone resorption by modulating osteoclast formation and function *in vitro*. Although isolated bone cell cultures are useful models to directly test the effects of purified CLA on bone formation and resorption, they do not account for differences in CLA metabolism or bone physiology, which are both affected by a number of factors, including age, sex, diet, and genetic variability. Findings from this study warrant further investigation of the effects of the naturally occurring 9*cis*,11*trans* isomer of CLA on bone formation in humans.

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