Journal of Cellular Biochemistry 85:825–836 (2002)

Leptin Stimulates Human Osteoblastic Cell Proliferation, De Novo Collagen Synthesis, and Mineralization: Impact on Differentiation Markers, Apoptosis, and Osteoclastic Signaling

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Abstract Anabolic hormones, mechanical loading, and the obese protein leptin play separate roles in maintaining bone mass. We have previously shown that leptin, as well as its receptor, are expressed by normal human osteoblasts. Consequently, we have investigated how leptin affects proliferation, differentiation, and apoptosis of human osteoblasts. Iliac crest osteoblasts, incubated with either leptin (100 ng/ml), calcitriol (1,25(OH)$_2$D$_3$; 10^{-9} M) or 1–84 human parathyroid hormone (PTH; 10^{-8} M), were cultured for 35 consecutive days and assayed for expression of various differentiation-related marker genes (as estimated by RT-PCR), de novo collagen synthesis, proliferation, in vitro mineralization, and osteoclast signaling. The effects of leptin on protection against retinoic acid (RA; 10^{-7} M) induced apoptosis, as well as transition into preosteocytes, were also tested. Leptin exposure enhanced cell proliferation and collagen synthesis over both control condition and PTH exposure. Leptin inhibited in vitro calcified nodule production after 1–2 weeks in culture, however, subsequent to 4–5 weeks, leptin significantly stimulated mineralization. The mineralization profile throughout the entire incubation period was almost undistinguishable from the one induced by PTH. In comparison, 1,25(OH)$_2$D$_3$ generally reduced proliferation and collagen production rates, whereas mineralization was markedly enhanced. Leptin exposure (at 2 and 5 weeks) significantly enhanced the expression of TGFβ, IGF-I, collagen-Iα, ALP, and osteocalcin mRNA. Leptin also protected against RA-induced apoptosis, as estimated by soluble DNA fractions and DNA laddering patterns subsequent to 10 days of culture. The expression profiles of *Bax*-α and *Bcl-2* mRNAs indicated that leptin per se significantly protected against apoptosis throughout the entire incubation period. Furthermore, the osteoblast marker OSF-2 was diminished, whereas the CD44 osteocyte marker gene expression was stimulated, indicating a transition into preosteocytes. In terms of osteoclastic signaling, leptin significantly augmented the mRNA levels of both interleukin-6 (IL-6) and osteoprotegerin (OPG). In summary, continuous leptin exposure of iliac crest osteoblasts, promotes collagen synthesis, cell differentiation and in vitro mineralization, as well as cell survival and transition into preosteocytes. Leptin may also facilitate osteoblastic signaling to the osteoclast.
J. Cell. Biochem. 85: 825–836, 2002. © 2002 Wiley-Liss, Inc.

Key words: proliferation; collagen synthesis; mineralization; differentiation; apoptosis; osteoclast stimulation

Obesity yields an unequivocally protective effect on bone mass, and has been ascribed to high body fat content in relation to enhanced leptin plasma levels [Klein et al., 1998]. Thus, leptin, which is a gene product synthesized and released from adipocytes [Zhang et al., 1994; Stephens et al., 1995], may serve as an important signal to modulate osteoblastic function. In the natural leptin knock-out model, *ob*/*ob* mice, leptin administration increases bone density and mineral content, length of limbs and brain mass [Steppan and Swick, 1999], as well as total body bone area, bone mineral contents, and density [Steppan et al., 1999].

Grant sponsor: Anders Jahre Medical Research Foundation, Norway; Grant sponsor: Freia-Marabou Nutritional Research Foundation, Norway; Grant sponsor: Norwegian Society for Fighting Cancer, Norway.

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Received 11 December 2001; Accepted 30 January 2002
DOI 10.1002/jcb.10156

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Furthermore, total body bone mass, as measured by DXA-technology, and bone area in bone cross sections, correlate positively to serum leptin [Matkovic et al., 1997]. Hence, it was asserted that leptin may enhance periosteal envelope expansion in young women and protect against osteoporosis and osteoarthritis. Additionally, it was shown that leptin enhanced endocortical bone formation in obese *ob*/*ob* mice [Liu et al., 1997], and it was demonstrated that leptin increased the number of human bone nodules in vitro within a dose range of 1–100 ng/ml [Iwaniec et al., 1998]. Most importantly, it was shown that leptin preferentially induced differentiation of stromal cells into osteoblasts [Thomas et al., 1999; Marie et al., 2000] and chondrocytes, but also adipocytes [Marie et al., 2000], depending on incubation conditions.

However, some researchers [Anselme et al., 2000; Ducy et al., 2000; Fleet, 2000] failed to identify leptin receptors in osteoblasts, and advocate a strict neuroendocrine and indirect regulation of bone metabolism and function by leptin. The contradicting in vivo results provide a need for more investigation. Recently, we reported that leptin and its receptors are expressed by primary cultures of human iliac crest osteoblasts as demonstrated by reverse transcriptase polymerase chain reaction (RT-PCR) analysis and immunocytochemistry using fluorescent antibodies [Reseland et al., 2001]. Furthermore, it has been demonstrated that differentiated human mesenchymal stem cells express leptin and leptin receptor isoforms, as well as leptin-induced phosphorylation of STAT3 [Bassilana et al., 2000]. Hence, leptin may exert direct effects on osteoblasts, thereby modulating osteoblast recruitment, differentiation, and eventually bone modeling and remodeling.

Consequently, we have investigated how exogenously added leptin affects proliferation, matrix deposistion, and mineralization in human osteoblasts if the iliac crest. Secondly, we report the effect of leptin on osteoblast-to-osteocyte differentiation and programmed cell death, and finally, we demonstrate the impact of leptin on osteoblast-derived factors known to affect osteoclastic bone resorption.

MATERIALS AND METHODS

In Vitro Culturing of Osteoblastic Cells

Isolation of hOB cells (human osteoblasts) was performed essentially as described by Robey and Termine [1985]. Trabecular bone specimens (caput femoris and iliac crest) were obtained from osteoarthritic patients without malignant disease undergoing hip surgery. Soft connective tissue and periosteal and cortical bone were removed, and the remaining trabecular bone was minced and extensively washed with phosphate buffered saline (PBS) to remove bone marrow cells. The bone fragments, approximately 3 mm^3, were then digested at 37°C with 1 mg/ml type H bacterial collagenase (Sigma, St. Louis, MO, USA) in DMEM/F 12 (Gibco, Paisly, UK). After 2 h, the released cells were discarded and the remaining bone fragments were extensively washed with DMEM/F12 containing 10% fetal calf serum (FCS). The fragments were seeded into 25 cm^2 tissue culture flasks (Costar, Cambridge, MA, USA) and incubated at 37°C in a humidified atmosphere with 5% CO_2. The culture medium DMEM/F 12 was supplemented with 10% FCS, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, 1 mM pyruvate, and 2 mM glutamine. The medium was changed weekly until confluent cell monolayers were obtained after 4–6 weeks of incubation. Confluent cells (10,000 cells per cm^2) were detached with trypsin (2.5%) and EDTA (0.02%) and subcultured (dilution 1:4) further with an in vitro life span of 35–44 days.

Bone cultures from three different donors were used for the experiments in this study. In all the experiments with primary osteoblasts, the cells were cultured for up to 35 days in the absence or presence of recombinant leptin, 100 ng/ml (Biomol Research Laboratories, PA, USA), calcitriol ($1,25(OH)_2D_3$), 10^{-9} M (Sigma) or human 1–84 PTH, 10^{-8} M (Sigma). When cells were tested for incorporation of labeled thymidine or proline, they were detatched by using trypsin-EDTA and diluted 1:10 before passage. Osteoblasts cultured to facilitate mineralization were exposed to β-glycerophosphate and ascorbic acid in the ambient medium.

Rate of Cell Proliferation

^{3}H-thymidine incorporation: cells were plated at 1,000 cells per milliliter per well and exposed to 0.1 μCi of ^{3}H-thymidine for 60 min at 12-h intervals during 2 days at day zero, 14 and 35 of the total monolayer culturing period [Gordeladze et al., 1997]. Subsequent to each incubation period, the cells were washed three times with ice-cold PBS and solubilized with 0.1% SDS. Material precipitated by 20% TCA

was dissolved in 1 N NaOH and neutralized with 1 N HCl. Radioactivity was counted for 10 min in a liquid scintillation counter (Packard 1900 TR, Packard, Chicago, IL, USA).

De Novo Collagen Synthesis

Cells were pulsed with 5 μCi/ml of ^{3}H-proline for the final 4 h of culture at each time interval (every 7 days between day 0 and 35 of continuous monolayer culture). The medium was removed and the cells scraped into extraction buffer (1 M NaCl, 2.25 mM EDTA, 1 mM N-ethyl-maleimide, and 0.2 mM phenylmethylsulfonylfluoride). The medium and cells were pooled and sonicated and the protein precipitated with 15% TCA [Fall et al., 1994]. After repeated washing, the pellets were dissolved in 0.5 M NaOH and an aliquot digested with purified bacterial collagenase. Water-soluble radioactivity was counted for 10 min in a liquid scintillation counter (Packard 1900 TR). The amount of collagen synthesized was estimated and related to the number of collagen-producing cells in culture (i.e., total amount of DNA present). DNA contents were assayed in the cell debris/precipitated material remaining in each incubation vessel by the diphenylamine (DPA) method [Kissane, 1958]. Freshly made DPA-solution aliquots of 0.25 ml were added to each well, and optical density at 580 nm was read after 36 h on a Pharmacia LKB 4054 Ultraspec spectrophotometer (Pharmacia, Sweden). The amounts of DNA present in each well were calculated against a salmon sperm DNA standard curve.

In Vitro Mineralization

Osteoblasts were grown in RPMI 1640 medium with L-glutamine containing standard levels of penicillin, streptomycin, fungizone, and tylocine. For 35 days, this medium was fortified with β-glycerophosphate (10 mM) and ascorbic acid (50 μg/ml). At each assay interval (every 7 days), the cells were rinsed three times with PBS and fixed with 95% methanol for 30 min. Subsequently, the cells were stained with 1% alizarin red S at pH 6.4 for 5 min and washed with distilled water as previously described [Dahl, 1999]. Images of the mineralized nodules were obtained with a Zeiss Standard microscope (Carl Zeiss, Oberhochen, Germany) equipped with a CCD video camera (Hamamatsu C3077, Hamamatsu, Japan), and stored on a Macintosh computer running NIH Image software. Percent surface covered by mineral was estimated using a Zeiss I integrating eyepiece with seven parallel lines. Four hundred intersections between lines and mineralized surface were counted [Jablonski et al., 2000].

mRNA Isolation and Semi-Quantitative RT-PCR

Osteoblasts were lysed in lysis/binding buffer (100 mM Tris-HCl pH 8.0, 500 mM LiCl, 10 mM EDTA pH 8.0, 0.5 mM DTT, 1% SDS). MessengerRNA was isolated using magnetic beads (oligo $(dT)_{25}$) as described by the manufacturer (Dynal AS, Oslo, Norway). For the analysis of leptin and leptin receptor mRNAs, beads containing mRNA were resuspended in 10 mM Tris-HCl pH 8.0 and stored at −70°C until use. The GeneAmp EZ rTth RNA PCR kit (Perkin Elmer, Applied Biosystems, Foster City, CA) was used for the RT-PCR, and 2 μCi ^{32}P-dCTP was added for each reaction. Temperature cycles were as follows: 60°C for 30 min, 94°C for 1 min followed by 30–34 cycles at 94°C for 30 sec, and 60°C for 1.5 min. At the end, the samples were incubated at 60°C for 7 min. A 10 μl aliquot of the reaction mixture was electrophoresed on 2% agarose gels, stained with ethidium bromide, excised and counted for 1 min in a liquid scintillation counter (Packard 1900 TR).

As for the analysis of osteoblast/osteocyte cell markers, cells were pelleted at 700*g*, washed in PBS, and frozen at −80°C in aliquots of 4×10^6 cells. Subsequently, frozen cells were lysed in lysis/binding buffer and mRNA isolated using Dynabead (Dynal). After annealing and thorough washing of the beads, mRNA was eluted in 40 μl DEPC dH_20 at 65°C. The mRNA-containing solution was applied directly to obtain a first strand cDNA using the Pharmacia Biotech kit (with random hexamer primers and Moloney murine leukemia virus reverse transcriptase). Incubation conditions were 37°C for 60 min. The PCR-amplification reaction contained 10 μl of the cDNA mixture, 15 pmoles of sense and antisense primers, 2 μCi of ^{32}P-dCTP (3000 Ci/mmol), 2.5 mM Mg^{2+}, and 2.5 U of Taq polymerase. The cycling profile was as follows: denaturing at 94°C (5 min) followed by 20–40 cycles of annealing at 59°C (30 sec), primer extension at 72°C (45 sec), and denaturing at 94°C (30 sec). Finally, one cycle (3 min) of extension completed the reaction. A 10 μl aliquot of the reaction mixture was applied on 6% TB PAGE gel (Novex, Invitrogen, Carlsbad, CA, USA), stained with ethidium bromide, excised

and counted for 1 min as described above. The primers were selected by using Primer Analysis, Oligo™ Version 4.0 software (National Biosciences, Plymouth, MN, USA) and designed to give optimal annealing at 59°C.

Relative abundance of phenotype marker mRNAs was calculated as glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA ratios. Oligonucleotide sequences of sense and antisense primers were as follows:

Product designation	Estimated size (bp)	Sense and antisense primer sequences
G3PDH	452	5′-ACCACAGTCCATGCCATCAC-3′
		5′-TCCACCACCCTGTTGCTGTA-3′
Hormone sensitive lipase (HSL)	320	5′-AGGTGTTCGGGAACAGGCACTGG-3′
		5′-CGCCCTCAAAGAAGAGCACTCCT-3′
Transforming growth factor β (TGFβ)	363	5′-ACCGGCCTTTCCTGCTTCTCAT-3′
		5′-TGGGCTTGCGGCCCACGTAGTA-3′
Insulin like growth factor I (IGF-I)	533	5′-AGAGCCTGCGCAATGGAATAAA-3′
		5′-GGGTCTTGGGCATGTCGGTGT-3′
Collagen-I (α1)	306	5′-GCAAGAACCCCAAGGACAAGAG-3′
		5′-TCGTGCAGCCATCGACAGTGAC-3′
ALP	341	5′-CACGGGCACCATGAAGGAAAAG-3′
		5′-TGGCGCAGGGGCACAGGAGACT-3′
Osteocalcin	257	5′-GGCAGCGAGGTAGTGAAGAGAC-3′
		5′-GGCAAGGGGAAGAGGAAAGAAG-3′
Osteoblast specific factor (OSF-2)	415	5′-TGGAAGGGATGAAAGGCTGC-3′
		5′-CGGTGTTTACCACAGCAGGT-3′
CD44	430	5′-CAAGTTTTGGTGGCACACAGC-3′
		5′-GGTTAAGGAAGCTACCTGGC-3′
Bax-α	482	R&D Systems, RDP-43-025, lot no 0008025
Bcl-2	293	R&D Systems, RDP-44-025, lot no 9945153
IL-6	251	5′-CCCACACAGACAGCCACTCACCTC-3′
		5′-ATACCTCAAACTCCAAAAGACCAG-3′
OPG	538	5′-CCTTGCCCTGACCACTACTACACA-3′
		5′-GCCGTTTTATCCTCTCTACACTCT-3′

Estimation of Soluble DNA

Cells were washed twice with 2 ml PBS. Subsequently, they were added 0.5 ml of lysis buffer (10 mM Tris-HCl, 10 mM EDTA, and 0.5% Triton X-100, pH 8.0), scraped off the incubation plate, and sonicated in Braun Labsonic L (Braun Biotech International, Oberhausen, Germany) apparatus at energy setting 4. The sonicate was then centrifuged at 14.000*g* for 20 min at 4°C. The supernatant and pellet were separated, subsequently incubated with 0.5 ml lysis buffer containing 0.5 mg/ml RNase A for 1 h, and then with 0.4 mg/ml proteinase K for 1 h. After adding 0.1 ml 5 M NaCl and 0.6 ml isopropanol, the samples were incubated overnight at −20°C. Fragmented DNA was calculated as soluble DNA/total DNA [Shimabukuro et al., 1998]. The soluble DNA was separated by electrophoresis on 1.5% agarose gels and exhibited a ladder-like appearance.

Statistical computations

Levels of mRNA, estimated by RT-PCR, were assessed by the amount of radiolabeled dCTP incorporated into each product band appearing on PAGE gels, and considered significantly different ($P < 0.05$) when deviating more than 35%. This cut point represents an average of $2 \times$ SD of six replicate assays.

Functional assays were analyzed by parametric (Analysis of Variance, Student's *t*-test) and non-parametric (Kruskall–Wallis test, Wilcoxon rank test) at the level of $P = 2\alpha = 0.05$. Both sets of tests gave essentially the same results, and parametric testing is referred to.

RESULTS

Influence of Leptin on Osteoblasts Proliferation

Proliferation of osteoblasts was estimated as the rate of radioactive thymidine incorprated into TCA-precipitable material at each time interval of incubation (Fig. 1). Incubation with leptin (100 ng/mL) for 48 h at day 0 consistently ($P < 0.05$) enhanced thymidine incorporation by a factor of 2.2 ± 0.4 over control condition. After 14 and 35 days of incubation, the relative effect of leptin was reduced to 1.6 and 1.0, respectively. Parallel incubations with either calcitriol ($1{,}25(OH)_2D_3$; 10^{-9} M) or 1–84 human parathyroid hormone (PTH;

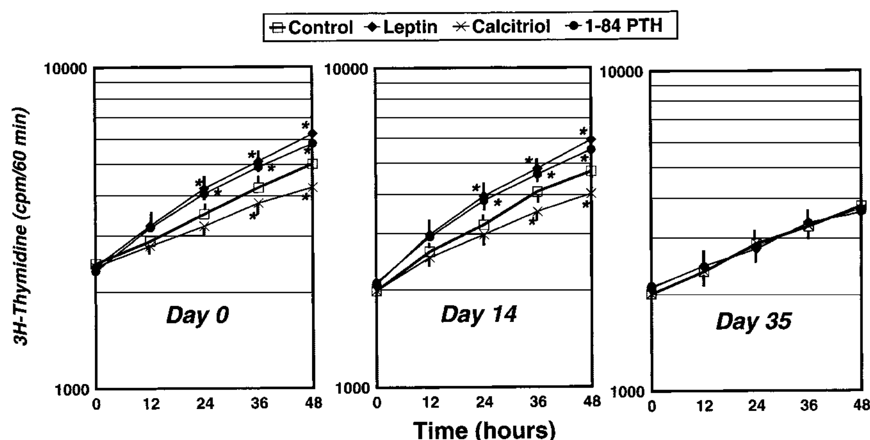

Fig. 1. Thymidine incorporation into human osteoblast DNA prior to and after 14 and 35 days of chronic incubation with leptin (100 ng/mL), calcitriol (10^{-9} M), and human 1–84 PTH (10^{-8} M). Incorporation of radiolabeled thymidine was performed during intervals of 60 min every 12 h of subconfluent cell cultures and presented as mean cpm/60 min incorporated into TCA-precipitable material $\pm 2 \times$ SD of four replicate measurements. *Indicates values significantly different from control levels ($P < 0.05$, Student's *t*-test). Cell proliferation rates (i.e., population doubling time $= t_d$) were estimated according to standard kinetic analysis and were as follows: day 0: control (48.6 h), leptin (42.5 h), calcitriol (54.3 h), PTH (44.5h); day 14: control (43.4 h), leptin (40.2 h), calcitriol (50.3 h), PTH (41.2h); day 35: all treatments (51.6 h).

10^{-8} M) either reduced ($P < 0.05$) or enhanced ($P < 0.05$) osteoblastic proliferation rates as compared to control incubation. The effect of leptin grossly mimicked that of 1–84 human PTH (10^{-8} M).

Impact of Leptin on De Novo Collagen Synthesis

De novo collagen synthesis was estimated as TCA-precipitable radioactive proline relative to cell DNA over a period of 4 h at each incubation time interval (0–35 days). Figure 2 demonstrates that de novo collagen synthesis diminished successively from approximately 105 cpm/μg DNA at day 0 to some 20 cpm/μg DNA at days 28–35 of incubation. Exposure to leptin (100 ng/mL) for 7 days raised ($P < 0.05$) the rate of collagen synthesis to 235 cpm/μg. However, extended incubation sustained ($P < 0.05$) the level at around 135 cpm/μg. Parallel incubations with calcitriol (10^{-9} M) and 1–84 human PTH (10^{-8} M) reduced ($P < 0.05$) and enhanced ($P < 0.05$), respectively, de novo collagen syntehesis as compared to control levels.

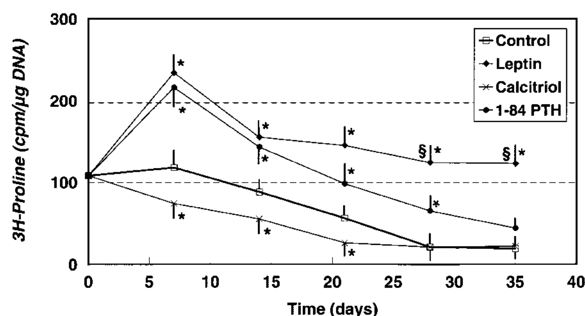

Fig. 2. Incorporation of radiolabeled proline (cpm/μg DNA) indicative of de novo collagen synthesis in human osteoblasts subsequent to continuous exposure to leptin (100 ng/mL), calcitriol (10^{-9} M), and human 1–84 PTH (10^{-8} M) for 35 days. All values represent means $\pm 2 \times$ SD of four replicate experiments where subconfluent cells were exposed to radioactivity for 4 h once a week through 35 days. *Indicates values significantly different from control levels ($P < 0.05$, Student's *t*-test). § Shows values significantly different between leptin and PTH exposure ($P < 0.05$, Student's *t*-test).

Effect of Leptin on In Vitro Mineralization

Mineralization of matrix proteins by osteoblasts was assessed by the presence of mineralizing noduli stainable by alizarin red (Fig. 3). Incubation with leptin (100 ng/mL) markedly reduced ($P < 0.05$) the cell monolayer surface covered by mineralized noduli during the first 14 days of incubation, however, at days 28 and 35, percentage mineralized surface had increased ($P < 0.05$) from a plateau of 12–28% and 33%, respectively. Parallel incubations with either calcitriol (10^{-9} M) or 1–84 human PTH (10^{-8} M) either consistently enhanced ($P < 0.05$) mineralization (calcitriol) or exerted a biphasic effect (PTH) on the percentage of mineralized surface as compared to control conditions. Again, the effect of leptin resembled that of 1–84 human PTH (10^{-8} M).

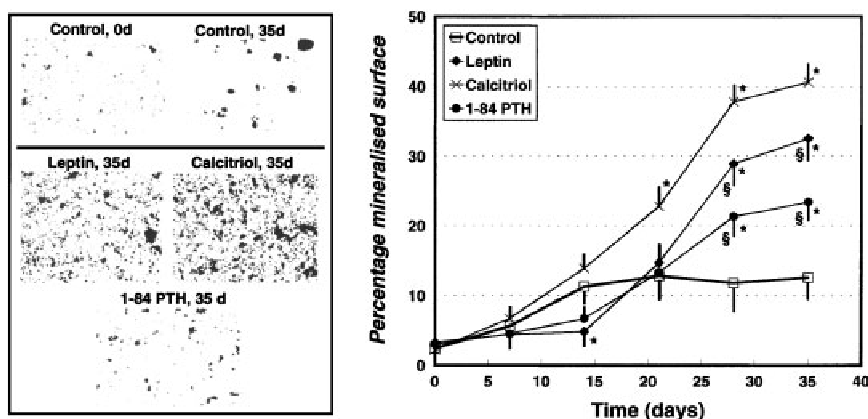

Fig. 3. The chronic effect of leptin (100 ng/mL), calcitriol (10^{-9} M), and human 1–84 PTH (10^{-8} M) on percent mineralized surface (estimated as mineralized cell nodules coloured by Alizarin S) in monolayer cultures of human osteoblasts in medium fortified with β-glycerophosphate for 35 days. Left panel: Microscopic pictures (400 × magnification) of mineralized nodules in cultures exposed to leptin, calcitriol, and PTH for 35 days. Right panel: Percentage of mineralized surface, as scored with a Zeiss I integrating ocular with seven parallel lines. All values represent means ± 2 × SD of four replicate measurements. *Indicates values significantly different from control levels ($P < 0.05$, Student's *t*-test). § Shows values significantly different between leptin and PTH exposure ($P < 0.05$, Student's *t*-test).

Effect of Leptin on Osteoblast Differentiation

Osteoblast differentiation, according to the various stages outlined by Stein and Lian [1993], was assessed by RT-PCR analysis of marker genes like TGFβ, IGF-I, collagen-Iα, ALP, and osteocalcin. As shown in Figure 4, exposure to leptin (100 ng/mL) for 14 days significantly ($P < 0.05$) stimulated the expression of collagen-Iα, ALP, and osteocalcin, respectively. Similar results were obtained subsequent to incubation with 1–84 human PTH (10^{-8} M), however, exposure to calcitriol (10^{-9} M) significantly enhanced ($P < 0.05$) the expression of ALP only.

By relating the expression of marker genes to that of the housekeeping gene G3PDH (Table 1), it appeared that TGFβ, IGF-I, collagen-αI, and ALP mRNAs were more readily expressed ($P < 0.05$) at day 14 as a result of exposure to leptin (100 ng/mL), whereas osteocalcin mRNA was markedly ($P < 0.05$) suppressed. At day 35, the effect of leptin on TGFβ, IGF-I, collagen-Iα gene expression partly subsided, while ALP mRNA expression was further enhanced ($P < 0.05$). Most significantly, however, leptin increased ($P < 0.05$) the relative osteocalcin mRNA level from 3.8 to 16.6. Furthermore, OSF-2 gene expression was diminished ($P < 0.05$) to approximately 55%, while CD44 mRNA amounts were augmented ($P < 0.05$) to some 250%, as compared to control level (Fig. 5).

Influence of Leptin on Apoptosis

Apoptosis was assessed by RA induced percentage of soluble DNA, corresponding DNA laddering pattern, and the expression of apoptosis-related genes (*bax-α and bcl-2*) in cultured osteoblasts. Exposure to RA (10^{-7} M) for 10 days in the absence or presence of leptin (100 ng/mL) resulted in a percentage of soluble DNA of 17.3 versus 3.1 ($P < 0.05$), respectively (Fig. 6). Exposure to leptin alone did not reduce ($P > 0.05$) the level of soluble DNA below control condition. A clear pattern of DNA laddering could be observed in the cells treated with RA (Fig. 6). Exposure to leptin for 14 and 35 days reduced ($P < 0.05$) the expression of *bax-α* to some 35% of control level, while the amount of *bcl-2* was markedly enhanced ($P < 0.05$) by a factor of 2.3–2.4, respectively (Fig. 7).

Impact of Leptin on Osteoclast-Signaling Markers Expressed by Osteoblasts

Osteoclast-signaling markers were mRNA species of interleukin-6 (IL-6) and osteoprotegerin (OPG). Exposure to leptin (100 ng/mL) for 14 and 35 days consistently enhanced ($P < 0.05$) the relative expression of IL-6 to 3.3 and 5.4, while that of OPG was increased ($P < 0.05$) to 3.7 and 4.2, respectively (Fig. 8).

DISCUSSION

The protective effect of obesity on osteoporosis is clinically established, but the complex

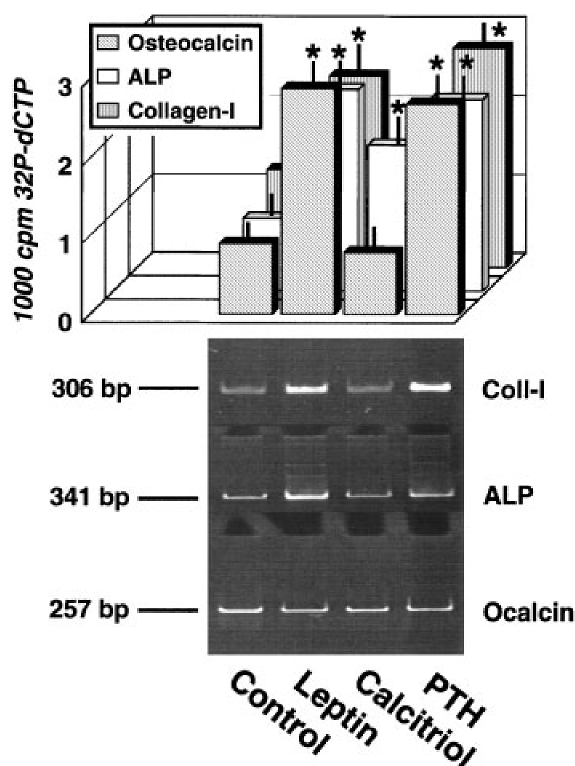

Fig. 4. The chronic effect of leptin (100 ng/mL), calcitriol (10^{-9} M), and human 1–84 PTH (10^{-8} M) on the levels of collagen-I, ALP, and osteocalcin mRNA levels in human osteoblasts incubated for 14 days in monolayer culture, as estimated by RT-PCR. The upper panel depicts the amount of radiolabeled dCTP (cpm) incorporated into the PCR product. Values are given as means ± ranges of three different experiments. *Indicates values significantly different from control levels (i.e., differing more than twice the inherent variance = 2 × SD of control expression performed on six occasions). The lower panel depicts ethidium bromide stained DNA bands separated on a 6% PAGE gel with estimated PCR product sizes of 306 bp (Collagen-I), 341 bp (ALP), and 257 bp (osteocalcin, respectively).

integration of factors and mechanisms involved remains to be elucidated. Several observations indicate that the leptin–leptin receptor axis is important for cell proliferation in many tissues. In addition to their regulation of metabolic rate and food intake, leptin and the leptin receptor seem to be important for fetal growth, sexual maturation, proliferation of certain T-cells, haematopoietic stem cells, and pituitary adenoma cells [Lord et al., 1998; Shimon et al., 1998]. For the first time, we recently demonstrated that primary human osteoblasts transcribe, translate, and secrete leptin [Reseland et al., 2001]. Leptin was previously demonstrated in various fetal bone tissues [Hoggard et al., 1997], whereas our results indicated an abundant expression of leptin in osteoblasts

TABLE 1. RT-PCR Analysis of Various mRNA Species in Human Osteoblasts Relative to Glycerol 3-Phosphate Dehydrogenase (G3PDH) Expression Subsequent to Continuous Exposure to Leptin (100 ng/ml), Calcitriol (10^{-9} M) and 1–84 PTH (10^{-8} M) for 14, and 35 Days, Respectively

Messenger RNA species	Incubation with	Day 14	Day 35
TGFβ	Control	1.1 ± 0.2	0.3 ± 0.2
	Leptin	3.3 ± 0.5*	1.8 ± 0.3*
	Calcitriol	0.9 ± 0.2	0.3 ± 0.1
	1–84 PTH	2.3 ± 0.3*	1.7 ± 0.2*
IGF-I	Control	1.3 ± 0.1	0.6 ± 0.1
	Leptin	2.5 ± 0.3*	2.0 ± 0.2*
	Calcitriol	0.7 ± 0.2*	0.5 ± 0.1
	1–84 PTH	2.2 ± 0.3*	1.7 ± 0.3*
Collagen-Iα	Control	0.7 ± 0.3	0.2 ± 0.2
	Leptin	2.2 ± 0.3*	2.1 ± 0.2*
	Calcitriol	0.8 ± 0.1	0.6 ± 0.2
	1–84 PTH	2.0 ± 0.2*	1.6 ± 0.2*
ALP	Control	2.2 ± 0.4	1.7 ± 0.2
	Leptin	3.3 ± 0.3*	4.4 ± 0.3*
	Calcitriol	3.5 ± 0.4*	2.8 ± 0.3*
	1–84 PTH	2.8 ± 0.3*	2.1 ± 0.3
Osteocalcin	Control	3.3 ± 0.4	3.8 ± 0.4
	Leptin	1.5 ± 0.2*	16.6 ± 2.8*
	Calcitriol	8.8 ± 1.5*	14.4 ± 2.0*
	1–84 PTH	2.1 ± 0.3*	10.5 ± 1.5*

The PCR reactions were conducted in the presence of radiolabeled dCTP and mRNA expressions calculated as ratios to the expression of G3PDH mRNA. Control values at time zero was arbitrarily set as 1.0. Tabulated values represent means ± ranges of three different experiments. *Indicates values significantly different from control levels (i.e., differing more than twice the inherent variance = 2 × SD of control expression performed on six occasions).

isolated from adult bone tissue. In this work, we advocate certain functions and biological roles for the leptin–leptin receptor axis in osteoblasts and bone remodeling. The results clearly indicate that leptin promotes bone formation by

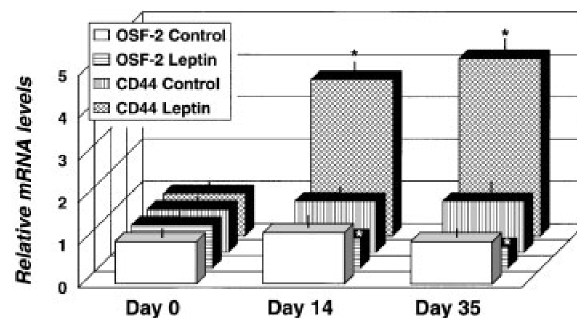

Fig. 5. The effect of continuous leptin (100 ng/mL) exposure on OSF-2 and CD44 mRNA levels in human osteoblasts incubated for 14 and 35 days in monolayer culture, as estimated by RT-PCR. Control values at time zero were arbitrarily set as 1.0. Tabulated values represent means ± ranges of three different experiments. *Indicates values significantly different from control levels (i.e., differing more than twice the inherent variance = 2 × SD of control expression performed on six occasions).

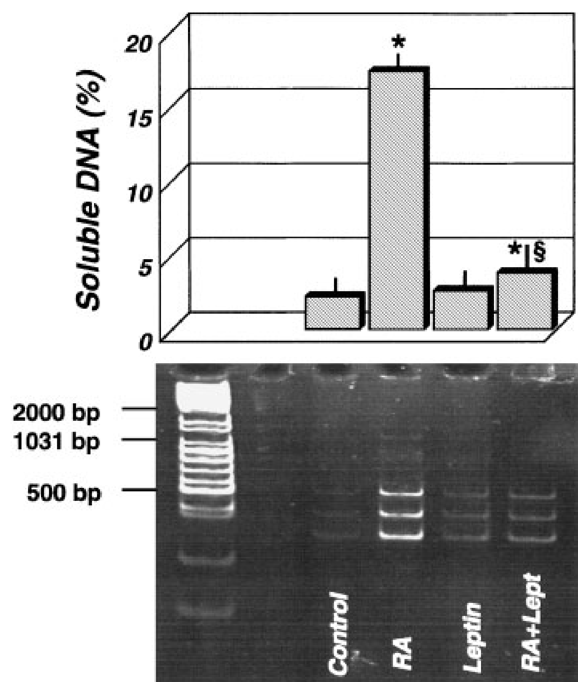

Fig. 6. Percentage soluble DNA and its laddering pattern in human osteoblasts exposed to either all trans RA (10^{-7} M), leptin (100 ng/mL) or RA + leptin for 10 consecutive days in monolayer culture. Upper panel: Percentage soluble DNA represented by values $\pm 2 \times$ SD of four replicate measurements. *Indicates values significantly different from control levels ($P < 0.05$, Student's *t*-test). § Shows values significantly different between leptin and leptin plus RA exposure ($P < 0.05$, Student's *t*-test). Lower panel: Soluble DNA aliquots run on a 6% PAGE gel and visualized by ethidium bromide and UV light.

enhancing human osteoblast proliferation, de novo collagen synthesis, and in vitro mineralization, which should be construed as a direct impact on matrix deposition and increased in vivo bone mineral contents.

It is well known that osteoblasts pass through a sequence of events controlled by hormones and transcriptional factors ensuring proper development of phenotype and functional properties until the osteoblasts enter the osteocyte phenotype and/or undergo apoptosis [Stein and Lian, 1993; Lian et al., 1999]. During their life cycle, the osteoblasts proliferate, deposit matrix proteins, and mineralize the matrix until they turn into osteocytes believed to constitute a mechanosensor mesh giving feedback to the osteoblast to initiate bone modeling or remodeling necessary for the making or remaking of proper bone architecture and strength [Stein and Lian, 1993; Manolagas and Jilka, 1995; Aubin and Liu, 1996; Stein et al., 1998; Lian et al., 1999]. In this context, it should be noted

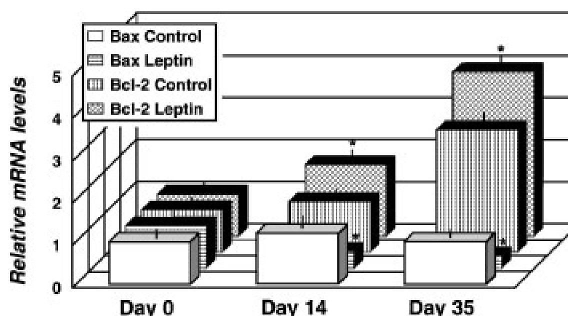

Fig. 7. The chronic effect of leptin (100 ng/mL) on *Bax* and *Bcl-2* mRNA levels in human osteoblasts incubated for 14 and 35 days in monolayer culture, as estimated by RT-PCR. Control values at time 0 were arbitrarily set as 1.0. Tabulated values represent means $\pm$ ranges of three different experiments. *Indicates values significantly different from control levels (i.e., differing more than twice the inherent variance $= 2 \times$ SD of control expression performed on six occasions).

that the osteoblasts continuously proliferated and synthesized collagen, but had to be cultured for at least 3–4 weeks subsequent to passage from confluent primary cultures to respond positively to leptin and parathyroid hormone (PTH) in terms of mineralization. After 5 weeks of growth, the osteoblasts stopped proliferating and mineralization was high with a concomitantly high level of mRNA for osteocalcin. Our previous report [Reseland et al., 2001] suggested that leptin expression appeared at a late osteoblast/early preosteocyte stage, and leptin expression from clonal human osteoblasts did not express leptin until 3–4 weeks in differentiating medium [unpublished results].

It seems as if leptin expression is apparent in mesenchymal stem cells, suppressed in pro-

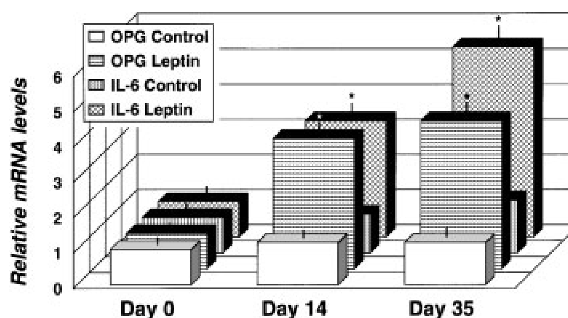

Fig. 8. The impact of leptin (100 ng/mL) on OPG and IL-6 mRNA expression in human osteoblasts maintained for 14 and 35 days in monolayer culture, as estimated by RT-PCR. Control values at time 0 were arbitrarily set as 1.0. Tabulated values represent means $\pm$ ranges of three different experiments. *Indicates values significantly different from control levels (i.e., differing more than twice the inherent variance $= 2 \times$ SD of control expression performed on six occasions).

liferating osteoblasts, and then reappears in late osteoblasts, in order to locally enhance recruitment of cells to become osteoblasts and to facilitate matrix deposition and mineralization. This is consistent with the sequential appearance of gene products ensuring cell proliferation/collagen synthesis, alkaline phosphatase (ALP) production and osteocalcin secretion. Apparently, leptin mimics the effect of PTH on late osteoblasts, whereas calcitriol (1,25 $(OH)_2D_3$) counteracts most of the early effects of leptin on proliferation and collagen synthesis. Thus, in the stages where leptin is produced locally in bone marrow and bone tissue, it may act synergistically with humoral factors to optimize bone mass and mineral contents.

The lack of leptin in *ob*/*ob* mice, who are homozygous for the obese gene, results in aberrations in the regulation of glucose and fat metabolism, as well as reduced stature. Leptin administration leads to a significant increase in femoral length, total body area, as well as bone mineral content and bone density [Liu et al., 1997; Matkovic et al., 1997; Iwaniec et al., 1998; Steppan et al., 1999]. Other reports, however, assert that leptin inhibits bone formation through a central nervous regulatory loop [Anselme et al., 2000; Ducy et al., 2000; Fleet, 2000], claiming that there is no leptin signaling in osteoblastic cells. Our findings are substantiated by the very recent report by Bassilana et al. (2000), demonstrating that human mesenchymal stem cells, when differentiated, express leptin and leptin receptor isoforms, as well as leptin-induced phosphorylation of STAT3. In the light of the localization of leptin and its receptor isoforms in osteoblastic cells, it is not surprising that an accumulating number of publications demonstrate that leptin stimulates bone formation [Liu et al., 1997; Goulding and Taylor, 1998; Iwaniec et al., 1998; Huang and Li, 2000; Steppan et al., 2000], and that circulating leptin levels may be associated with bone mass in young individuals [Matkovic et al., 1997; Klein et al., 1998; Ballabriga, 2000]. Finally, human bone marrow adipocytes have been shown to synthesize leptin [Laharrague et al., 1998]. Leptin gene expression is inhibited by cytokines and tumor necrosis factor α (TNFα) [Laharrague et al., 2000], and may thus contribute to the proliferation and differentiation of stromal cells.

Leptin has proved to serve as an antiapoptotic agent in several cell systems including bone marrow stem cells and osteoblasts [Shimabukuro et al., 1998; Takeda et al., 1998; Konopleva et al., 1999]. Our results indicate that leptin may inhibit RA-induced DNA laddering and disruption of DNA in human osteoblasts. Furthermore, we demonstrate that osteoblasts exposed to leptin exhibit a reduced *Bax*-α/*Bcl-2* mRNA ratio, indicating that leptin in fact serves as an antiapoptotic agent [Gordeladze et al., 2001]. Among several factors responsible for induction of apoptosis are long-chain fatty acids (LCFA). This is called lipoapoptosis, and it may be responsible for the gradual depletion of pancreatic β-cells [Unger and Zhou, 2001] leading to non-insulin-dependent diabetes mellitus and insulin hypersecretion seen in patients suffering from metabolic syndrome X. In the rodent model of obesity, the Zucker diabetic fatty *fa*/*fa* rat, lipoapoptosis is associated with high levels of fatty acyl-CoA and LCFA-induced *Bcl-2* suppression [Shimabukuro et al., 1998a; Gordeladze et al., 2001; Unger and Zhou, 2001].

Administration of leptin to normal islets blocked lipoapoptosis, but had no effect on *fa*/*fa* islets because of a mutation in their leptin receptors [Shimabukuro et al., 1998b,c]. Thus, leptin produced by osteoblasts may potentially increase the utilization of LCFA locally in order to enhance survival of osteoblast precursors, as well as mature osteoblasts. The fourth developmental period demonstrated by mature osteoblast cultures is characterized by collagenase gene expression, while Type I collagen gene expression is still elevated. However, apoptotic activity occurs (through the expression of *Bax*), and compensatory proliferative activity is noticeable [Gerstenfeld et al., 1987; Shalhoub et al., 1989; Aubin et al., 1993; Owen et al., 1993; Stein and Lian, 1993; Lynch et al., 1994; Lian et al., 1999]. When osteoblasts have completed their bone-forming function, some 30–50% of the cells are entrapped in the bone matrix and become osteocytes. Induction of apoptosis in osteoblasts by TNF exposure or serum starvation is associated with an increased ratio of *Bax*/*Bcl-2* protein. However, both transforming growth factor β (TGFβ) and the interleukin IL-6 proved to be antiapoptotic [Bellido et al., 1998; Jilka et al., 1998]. Finally, it has been demonstrated that osteoblasts, which undergo apoptosis due to serum starvation, exhibit an abrupt decline in mRNA species for type I collagen, ALP, and osteocalcin prior to apoptotic signs

like chromatin condensation, membrane budding and nucleosomal DNA degradation [Ihbe et al., 1998]. The present report shows that leptin, possibly via its antiapoptotic potential, facilitates the transition of mature osteoblasts to preosteocytes. This assertion is based on the facts that osteocalcin gene expression is vastly increased at day 35 of incubation, and that the expression of the osteoblast marker OSF-2 and the osteocyte marker CD44 [Kato et al., 1997] are reciprocally modulated.

In the human fetus, it was shown that serum leptin correlated negatively to cross-linked carboxy-terminal telopeptide of Type-I collagen (ICTP, a marker of bone resoprtion) [Ogueh et al., 2000]. Furthermore, leptin treatment increased OPG mRNA and protein expression in peripheral blood mononuclear cells (PBMC) treated with hM-CSF and sRANKL (human macrophage colony stimulating factor; soluble receptor activator of NFκB ligand) [Udagawa et al., 1999]. It is also important to note that the osteoblast secretes factors, which stimulate the osteoclast to resorb bone [Machwate et al., 1995; Sumoy et al., 1995; McCabe et al., 1996; Banerje et al., 1997; Otto et al., 1997; Ryoo et al., 1997; Lian et al., 1999]. Among these are the interleukins IL-6 and IL-11 [Jilka et al., 1998; Manolagas, 1998; Lian et al., 1999]. Osteoblasts also synthesize and secrete OPG, a molecule, which belongs to the TNF receptor family, and may capture osteoprotegerin ligand (OPGL), which normally stimulates osteoclasts to initiate resorption [Simonet et al., 1997; Tsuda et al., 1997; Kwon et al., 1998; Yasuda et al., 1998; Yun et al., 1998; Aubin and Bonnelye, 2000]. Human osteoblasts exposed to leptin enhance their expression of OPG mRNA, while also concomitantly enhancing IL-6 expression. These results are consistent with the concept that leptin may protect against bone resorption by osteoclasts when osteoblasts deposit matrix protein and mineralize it, while at the same time initiating remodeling by activating osteoclasts localized to remodeling units.

CONCLUSION

Leptin modulates osteoblastic cell function, partly in a bimodal fashion, by acting in different stages of osteoblast recruitment and maturation, possibly by binding to differently expressed receptor isoforms. Leptin also appears to be antiapoptotic, facilitating the transition of osteoblasts into osteocytes. Finally, leptin is able to enhance the expression of factors, which modulate osteoclastic resorption.

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