

Leptin Acts on Human Marrow Stromal Cells to Enhance Differentiation to Osteoblasts and to Inhibit Differentiation to Adipocytes*

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ABSTRACT

Both bone mass and serum leptin levels are increased in obesity. Because osteoblasts and adipocytes arise from a common precursor in bone marrow, we assessed the effects of human recombinant leptin on a conditionally immortalized human marrow stromal cell line, hMS2-12, with the potential to differentiate to either the osteoblast or adipocyte phenotypes. By RT-PCR and Western immunoblot analysis, the hMS2-12 cells expressed messenger RNA (mRNA) and protein for the leptin receptor. Leptin did not affect hMS2-12 cell proliferation, but resulted in dose- and time-dependent increases in mRNA and protein levels of alkaline phosphatase, type I collagen, and osteocalcin, and in a 59% increase in mineralized matrix. Leptin increased

mRNA levels of lipoprotein lipase at 3 days, but decreased mRNA levels of adiponin and leptin at 9 days and decreased lipid droplet formation by 50%. Leptin did not affect the expression of *Cbfa1* or peroxisome proliferator-activated receptor- γ_2 , transcription factors involved in commitment to the osteoblast and adipocyte pathways, respectively. Thus, leptin acts on human marrow stromal cells to enhance osteoblast differentiation and to inhibit adipocyte differentiation. Our data support the hypothesis that leptin is a previously unrecognized, physiological regulator of these two differentiation pathways, acting primarily on maturation of stromal cells into both lineages. (*Endocrinology* 140: 1630–1638, 1999)

OSTEOBLASTS and adipocytes arise from a common precursor in bone marrow (1, 2), and the trabecular bone and adipose tissue content in bone marrow are inversely related in human disuse osteoporosis (3) and postmenopausal osteoporosis (4). Leptin was discovered as the product of the *ob* gene, which, when mutated, results in obesity in the *ob/ob* mouse (5). This 16-kDa protein is secreted mainly by white adipose tissue (6) and regulates food intake and body weight by negative feedback at the hypothalamic nuclei (7, 8). Recent studies have shown that in addition to its effects on the central nervous system, leptin acts through high affinity leptin receptors on cells in peripheral tissues (reviewed in Ref. 9). Leptin suppresses specific biochemical processes that contribute to lipid accumulation and adipocyte differentiation (10, 11). Leptin also stimulates hematopoietic precursor development directly (12, 13).

Several clinical studies have demonstrated that body fat and bone mass are directly related (14–17). Although mechanical loading may contribute to this relationship, the direct relationship remains regardless of whether the skeletal site is weight bearing (18), suggesting that other factors are also involved. Increased conversion of androgens to estrogens by peripheral aromatization in adipose tissue has been thought to be one possible causal mechanism (19). In addition, we have considered the possibility that leptin may be

the hormonal mediator relating fat mass and bone mass. Serum leptin levels are increased in obesity and correlate positively with fat mass (20). This observation led us to investigate the action of leptin on osteoblastic differentiation and function *in vitro*.

In this study, we evaluated the effects of recombinant human leptin on the conditionally immortalized human marrow stromal cell line, hMS2-12, with the potential to differentiate to either osteoblastic or adipocytic lineages (21). Our data show that leptin enhances osteoblastic differentiation of marrow progenitors and inhibits late adipocytic differentiation.

Materials and Methods

Reagents

Tissue culture media and reagents were purchased from either Sigma Chemical Co. (St. Louis, MO) or Life Technologies (Grand Island, NY). Tissue culture plasticware was obtained from Corning (Corning, NY). Molecular biology reagents and enzymes were purchased from Boehringer Mannheim (Indianapolis, IN). The RNA STAT-60 kit was purchased from Tel-Test, Inc. (Friendswood, TX). The Wizard PCR Preps DNA purification system was obtained from Promega Corp. (Madison, WI). $1\alpha,25$ -Dihydroxyvitamin D₃, [3 H]thymidine, and [α - 32 P]deoxy (d)-CTP were obtained from DuPont New England Nuclear (Boston, MA). L-Ascorbic acid phosphate was purchased from Wako Pure Chemical Industries Ltd. (Richmond, VA). Kits for the measurement of osteocalcin and procollagen protein were gifts from Metra Biosystem (Mountain View, CA). Leptin was provided by Eli Lilly & Co. (Indianapolis, IN).

Cell culture

The conditionally immortalized human marrow stromal (hMS) cell lines were established in our laboratory by transfecting the hMS cells with a gene coding for a temperature-sensitive mutant (tsA58) of simian virus 40 large T antigen (SV40LTA) (21). As previously reported (21), incubation of the cells at 34 C, the permissive temperature for SV40LTA, increases the rate of cell proliferation and inhibits differentiation until

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confluence. At 39.5 C, the restrictive temperature, SV40LTA is consistently inactive, little cell division occurs, and the cells begin to differentiate. Because the six cell lines that we characterized displayed a homogeneous phenotype (21), we used the hMS2-12 cell line for these studies.

hMS2-12 cells were maintained in a humidified atmosphere at 34 C in 5% CO₂ in α MEM containing 10% (vol/vol) heat-inactivated FBS (HI-FBS), geneticin (G418; 0.2 μ g/ml), and 1% stock penicillin (10,000 U/ml)-streptomycin (10,000 μ g/ml), hereafter termed standard growth medium. Medium was changed twice a week. To assess the effects of leptin on the shunting between adipocytic and osteoblastic lineages, the study required culture conditions in which the hMS cell lines have an equal propensity to differentiate toward either osteoblasts or adipocytes. Thus, as previously demonstrated (Gori, F., *et al.*, manuscript submitted for publication), all experiments were performed in a medium (hereafter termed standard differentiation medium) containing 10% HI-FBS, 10⁻⁸ M dexamethasone (DEX), 10⁻⁸ M 1,25-dihydroxyvitamin D₃, 10 mM β -glycerolphosphate, and 100 μ M L-ascorbate phosphate in the presence of freshly prepared leptin or vehicle (phosphate buffer disodium, 120 μ M final concentration; pH 7.5), unless otherwise indicated.

The preadipocyte cell line 3T3-L1 was used as a control for the expression of peroxisome proliferator-activated receptor- γ_2 (PPAR γ_2). Cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in α MEM, with nonessential amino acids and Earle's Balanced Salt Solution, and 10% calf serum.

Western immunoblot for leptin receptor

Western blot analysis for leptin receptor (OB-R) was performed using a rabbit polyclonal IgG epitope affinity-purified anti-OB-R antibody against the common form of OB-R (ABR, Golden, CO).

Cells were plated at a density of 2×10^4 cells/cm² in T75 flasks in standard growth medium and maintained for 4 days at 34 C. They were then washed twice in PBS and cultured in standard differentiation medium at 39.5 C, in the presence of 0.6 μ g/ml leptin or vehicle. After 6 days, cells were washed twice with PBS, and the pellet was suspended in electrophoresis buffer and electrophoresed in a 7.5% SDS-PAGE under reducing conditions, using a protein mixture (Amersham, Arlington Heights, IL) as standards. The blots were then electrotransferred onto a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). A hematopoietic cell line, K562, was used as a positive control.

The blots were blocked for 2 h in Tris-HCl phosphate buffer (TBS; pH 7.4) containing 0.1% (vol/vol) Tween-20 and 0.1% (wt/vol) BSA (blocking buffer), and then hybridized in blocking buffer with an anti OB-R antibody (1 μ g/ml) at 4 C. After overnight incubation the blots were washed twice with PBS containing 0.1% (vol/vol) Tween-20 and incubated in blocking buffer for 2 h with a peroxidase-conjugated affinity pure IgG goat antirabbit (1:10,000 final dilution). After three washes in TBS containing 0.1% (vol/vol) Tween-20, immunoreactive proteins were visualized using the ECL chemiluminescence detection kit (Amersham) according to the manufacturer's instructions.

Assessment of cell proliferation

Cell proliferation was assessed by [³H]thymidine incorporation. Cells were plated at a density of 2×10^4 cells/well in 24-well microtiter plates in standard growth medium. After 48 h at 34 C, cells were washed twice in PBS and incubated at 34 C for an additional 24 h in serum-free α MEM and 0.1% (wt/vol) BSA to synchronize the cell population. Cells were then incubated in standard differentiation medium in the presence of leptin (0.6 μ g/ml) or vehicle for 48 h at 34 or 39.5 C. To assess DNA synthesis, 1 μ Ci [³H]thymidine was added for the last 24 h of incubation. Cells were harvested by trypsinization, and [³H]thymidine was extracted by trichloroacetic precipitation and detected by scintillation counting.

Semiquantitative RT-PCR

Cells were plated at a density of 1.8×10^5 cells/well in six-well microtiter plates in standard growth medium and maintained for 4 days at 34 C. They were then washed twice in PBS and cultured for various time intervals in standard differentiation medium at 39.5 C in the presence of leptin (0.15–2.4 μ g/ml) or vehicle. Total cellular RNA was

extracted using the RNA STAT-60 kit following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 2 μ g total RNA in a 20- μ l reaction mix containing 4 μ l of 5 \times incubation buffer for AMV reverse transcriptase; 50 pmol poly(deoxythymidine)₁₅ primer; 20 nmol each of dATP, dCTP, dGTP, and dTTP; 20 U ribonuclease inhibitor; and 20 U AMV reverse transcriptase. The reaction time was 1 h at 42 C.

Aliquots of 1 μ l cDNA were amplified in a 25- μ l PCR mixture that contained 2.5 μ l of 10 \times Expand high fidelity PCR buffer with 15 mM MgCl₂; 5 pmol 5'- and 3'-oligo primers; 2.5 nmol each of dATP, dCTP, dGTP, and dTTP; 0.25 μ l [α -³²P]dCTP (10 μ Ci/ μ l); and 0.35 U Expand high fidelity Taq DNA polymerase. Each cDNA sample was amplified in duplicate PCR for each gene. Amplification reactions were performed in a GeneAmp 9600 thermal cycler (Perkin Elmer, Norwalk, CT), for the following cDNAs: adipin, bone/liver/kidney alkaline phosphatase (AP), type I collagen (Col I), core-binding factor- α 1 (Cbfa1), leptin, lipoprotein lipase (LPL), common region of OB-R variants, long form of OB-R, osteocalcin (OC), and PPAR γ_2 . The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as a control for RNA loading and variations in cDNA synthesis efficiency. After initial determination of the linear phase of amplification, reactions were performed for 28–35 cycles depending on product intensity, except for GAPDH, which was performed for 24 cycles. All PCR reactions were conducted by annealing at 55 C and ended in a 7-min incubation at 72 C. Primer sequences for these genes have been reported previously (21), except for adipin, leptin, PPAR γ_2 , and OB-R. A 251-bp cDNA fragment of adipin (sense, 5'-GGTCACCCAAGCAACAAAGT-3'; antisense, 5'-CCTCTGCGTTCAAG-TCATC-3'), a 227-bp cDNA fragment of leptin (sense, 5'-GCTTTGGCCCTATCTTT-TCT-3'; antisense, 5'-CACGTT-TCTGGAAGCAAC-3'), and a 390-bp cDNA fragment of PPAR γ_2 (sense, 5'-CAGTGGGGATG-CTCATAA-3'; antisense, 5'-CTTTTGGCAT-ACTCTGTGAT-3') were amplified for 30–35 cycles with denaturation at 94 C for 30 min, annealing at 55 C for 30 min, and extension at 72 C for 30 min. A 375-bp fragment from a region common to all OB-R variants (sense, 5'-TGTTGTGAATGTCTTGTGCC-3'; antisense, 5'-TACTCCAGTCACTCCAGATTCC-3') and a 240-bp fragment specific to the long form variant of the OB-R (sense, 5'-ATAGTTCAGTCAC-CAAGTC-3'; antisense, 5'-GTCCTGGAGAAGT-CTGATGTCC-3') were amplified, using the same conditions. Cbfa1 primers were as reported by Komori *et al.* for amplification of the mouse Cbfa1 gene (22); they amplified a 267-bp fragment starting at nucleotide 136 of the human cDNA sequence with 98% homology between the amplified fragment and human sequences.

PCR products were analyzed as described previously (21). Briefly, 9- μ l samples were electrophoresed on a 1.5% (wt/vol) agarose gel containing 0.01% (wt/vol) ethidium bromide. Visualized PCR product bands were excised from the gel, and radioactivity within gel slices was quantitated using a Beckman Coulter, Inc. LS600 scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Quantification of PCR product was normalized to the GAPDH PCR product. The cDNA from three separate RNA samples were analyzed for each gene and condition. The different gene products were purified using the Wizard PCR Preps DNA kit. For sequence analysis, approximately 150 ng of each purified cDNA fragment were added to 3.2 pmol of either 5'- or 3'-primer and analyzed in both directions in an automated DNA sequence analyzer.

Assays of bone-related proteins

AP activity. Cells were plated at a density of 2×10^4 cells/well in 48-well microtiter plates in standard growth medium and allowed to adhere for 4 days at 34 C. They were washed twice in PBS and further incubated in standard growth medium at 39.5 C in the presence of leptin (0.6 μ g/ml) or vehicle for 3, 6, and 9 days or in the presence of increasing doses of leptin (0.075–2.4 μ g/ml). AP enzyme activity was quantitated in cell lysate by spectrophotometric measurement of *p*-nitrophenol release at 37 C (23).

Measurement of Col I and OC proteins. Cells were plated at a density of 8×10^4 cells/well in 12-well microtiter plates in standard growth medium and allowed to proliferate for 4 days at 34 C. Cells were then washed twice in PBS and incubated at 39.5 C in standard differentiation medium in the presence of leptin (0.6 μ g/ml) or vehicle for 21 days. Medium was changed every 3 days and replaced in all conditions 24 h before harvest

FIG. 1. Expression of the OB-R. hMS2-12 cells were cultured in standard growth medium for 4 days at 34°C and then cultured at 39.5°C in the standard differentiation medium in the presence of leptin or vehicle for 6 days. A, Aliquots of cDNA synthesized from 2 μ g total RNA were amplified in a 25- μ l PCR. The expression of the common region of OB-R variants, the long form OB-R variant, and GAPDH was visualized on 1.5% (wt/vol) agarose gel containing 0.01% (wt/vol) ethidium bromide. B, Equal aliquots of total cell lysates (5×10^5 cells) were examined on Western blots probed with a rabbit polyclonal IgG epitope affinity-purified anti-OB-R antibody directed against the common form of OB-Rs. K562, a hematopoietic cell line, was used as a control. Antibody-protein complexes were visualized by chemiluminescence detection. A band representing the long form variant was readily detected at 39.5°C, but not at 34°C. The short form variant was present at both 34 and 39.5°C as well as in the K562 control cells. Leptin did not change the level of either form of the OB-R.

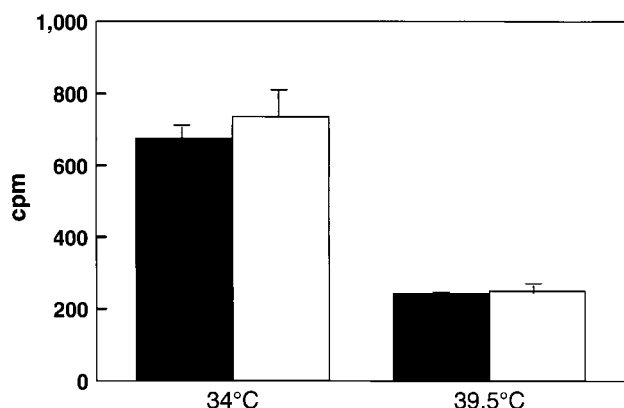
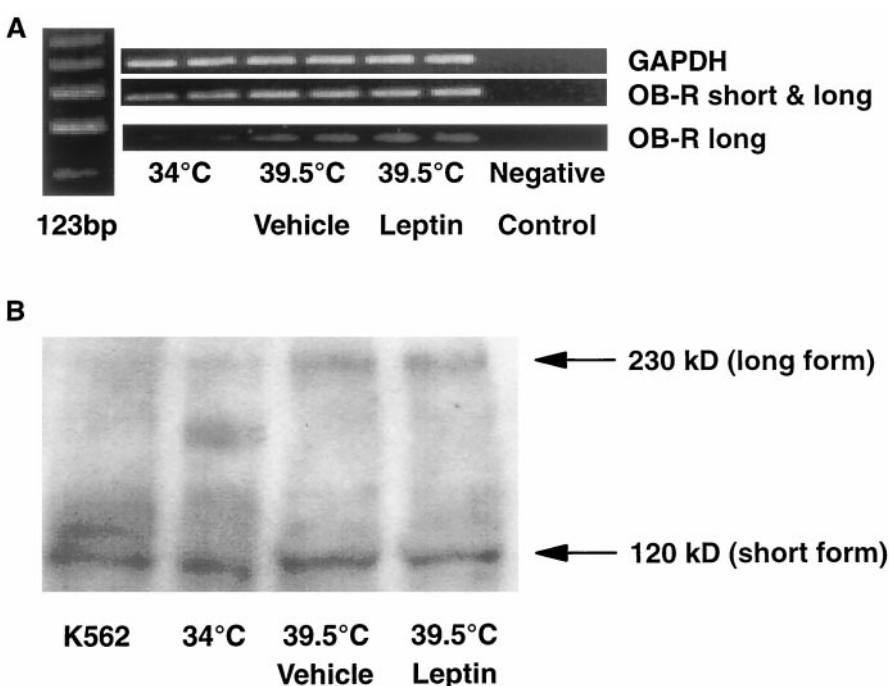


FIG. 2. Effect of leptin on cell proliferation. Cells were cultured in standard growth medium for 48 h at 34°C, followed by further 24 h in serum-free α MEM and 0.1% (wt/vol) BSA, and then incubated in standard differentiation medium in the presence of leptin at 0.6 μ g/ml (open bar) or vehicle (solid bar) for 48 h at 34 or 39.5°C. DNA synthesis was assessed by incorporation of [3 H]thymidine, which was added for the last 24 h of incubation. Results are expressed as the mean \pm SEM of quadruplicate determinations. The data shown are representative of five experiments. No significant differences were observed between control and leptin treatments at both permissive and restrictive temperatures.

with 1 ml α MEM containing 0.1% (wt/vol) BSA. Conditioned medium was collected from 12–21 days of culture and measured for Col I (Pro-lagen-C, Metra Biosystem, Mountain View, CA) and OC (Novocalcin, Metra Biosystem) proteins by enzyme-linked immunosorbent assay. Results were then normalized to total cellular protein values, as measured in cell lysates by the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA).

Assay of mineralized matrix formation

Cells were plated at a density of 8×10^4 cells/well in 12-well microtiter plates in standard growth medium and allowed to proliferate for 4 days at 34°C. They were then washed twice in PBS and incubated at

39.5°C in standard differentiation medium in the presence of leptin (0.6 μ g/ml) or vehicle for 21 days. Medium was changed every 3 days and replaced in all conditions 24 h before harvest with 1 ml α MEM containing 0.1% (wt/vol) BSA. After collecting conditioned medium, the extent of mineralized matrix was determined by Alizarin Red S staining. Briefly, cells were fixed in 70% ethanol for 1 h at room temperature, then washed with PBS and stained with 40 mM Alizarin Red S, pH 4.2, for 10 min at room temperature. Next, cell preparations were washed five times with deionized water and incubated in PBS for 15 min to eliminate nonspecific staining. The stained matrix was assessed using a Nikon Diaphot inverted microscope and was photographed using a Nikon 35-mm camera (Nikon, Tokyo, Japan). As described by Bodine *et al.* (24), Alizarin Red S staining was released from cell matrix by incubation in cetylpyridinium chloride for 15 min. The amount of released dye was quantified by spectrophotometry at 540 nm. Results were then normalized to total cellular protein values, as measured in cell lysate by the Bradford method (Bio-Rad Laboratories, Inc.).

Assessment of cytoplasmic lipid droplet formation

Cells were plated at a density of 5×10^4 cells/well in 12-well microtiter plates in standard growth medium and allowed to proliferate for 4 days at 34°C. Cells were then washed in PBS and incubated in standard differentiation medium at 39.5°C in the presence of leptin (0.6 μ g/ml) or vehicle for 6, 9, 12, and 15 days. Cytoplasmic inclusions of neutral lipids were assessed by Oil Red O staining. The percentage of Oil Red O-positive cells was determined by counting cells in 30 contiguous fields/well after random starts.

PPAR γ_2 expression by Western blot analysis

To evaluate the protein expression of PPAR γ_2 in the hMS2-12 cell line, Western blot analysis was performed using a rabbit polyclonal IgG epitope affinity-purified anti-PPAR γ_2 antibody (ABR). Cells were plated at a density of 2×10^4 cells/cm 2 in T75 flasks in standard growth medium and maintained for 4 days at 34°C. They were then washed twice in PBS and cultured in standard differentiation medium at 39.5°C in the presence of leptin (0.6 μ g/ml) or vehicle for 1 and 3 days. The 3T3-L1 preadipocyte cell line was grown to confluence and then cultured either in its standard medium or in the presence of insulin (1 μ M), 3-isobutyl-1-methylxanthine (200 μ M), and DEX (10^{-6} M; 3T3-L1 differentiation medium) for 6 days. Cells were washed twice with PBS, the pellets were suspended in electrophoresis buffer, and equal aliquots of total cell

lysates (50 μ g total protein) were electrophoresed in 7.5% SDS-PAGE under reducing conditions, using a protein mixture (Amersham) as standards. The blots were then electrotransferred onto a nitrocellulose membrane (Schleicher & Schuell, Inc.). They were blocked for 2 h in TBS (pH 7.4) containing 0.1% (vol/vol) Tween-20 and 5% (vol/vol) milk (blocking buffer) and then hybridized in blocking buffer with an anti PPAR γ_2 antibody (1 μ g/ml) at 4 C. After overnight incubation, the blots were washed twice with TBS containing 0.1% (vol/vol) Tween-20 and incubated in blocking buffer for 2 h with a peroxidase-conjugated affinity-purified IgG goat antirabbit (1:10,000 final dilution). Blotting with the secondary antibody alone was performed as a control for the specificity of the reagent. After three washes in TBS containing 0.1% (vol/vol) Tween-20, immunoreactive proteins were visualized using the ECL chemiluminescence detection kit (Amersham) according to the manufacturer's instructions.

Statistical analysis

All values are expressed as the mean \pm SEM. Two-sample Student's *t* test was used to evaluate differences between the stimulated sample and the respective control. Multiple measurement ANOVA was used for dose- and time-dependent differences. *P* < 0.05 was considered significant.

Results

Presence of OB-R

By RT-PCR, the OB-R gene was expressed in hMS2-12 cells at both 34 and 39.5 C, as assessed by primers common to the short and long forms of OB-R (Fig. 1A). The gene expression of the different OB-R variants was increased in cells cultured at 39.5 C, when the cells stop proliferating and differentiate, as assessed by semiquantitative RT-PCR (data not shown). By Western blot analysis (Fig. 1B), the short form of OB-R (~120 kDa) was present in hMS2-12 cells at both 34 and 39.5 C, whereas the long form of OB-R (~230 kDa) was present only at 39.5 C. We further evaluated gene expression of the long form OB-R in the hMS2-12 cells that were cultured for 6 days under either purely adipogenic conditions (*i.e.* α MEM containing 15% rabbit serum, 10^{-8} M DEX, 10^{-8} M 1,25-dihydroxyvitamin D $_3$, 200 μ M isobutylmethylxanthine, and 50 μ g/ml insulin) or purely osteogenic conditions (*i.e.* α MEM

containing 10% HI-FBS, 10^{-8} M DEX, 10^{-8} M 1,25-dihydroxyvitamin D $_3$, 10 mM β -glycerolphosphate, and 100 μ M L-ascorbate phosphate), as previously described (21). No difference was observed between these conditions as assessed by semiquantitative RT-PCR. A trend in decreased gene expression was induced under leptin treatment as the adipogenic characteristic of the medium increased (data not shown).

Effect of leptin on cell proliferation

[3 H]Thymidine incorporation was not significantly affected by leptin at either 34 or 39.5 C (Fig. 2).

Effect of leptin on osteoblastic differentiation

Expression of phenotype marker genes. Messenger RNA (mRNA) expression was assessed using semiquantitative RT-PCR. GAPDH mRNA expression remained constant with time in culture and dose of treatment. Cbfa1 is a gene expressed early during differentiation, and its product serves as a transcriptional activator of the commitment to the osteoblastic lineage (22, 25, 26). Its targeted disruption leads to a skeleton composed of cartilage rather than bone (22, 25, 26). Leptin did not consistently affect Cbfa1 mRNA levels over the interval of 30 min to 72 h, although we observed in some experiments a small increase of 30% after 1 day at 39.5 C compared with that in cultures with vehicle (see Fig. 8). However, after 3 days at 39.5 C, leptin dose dependently increased gene expression of early osteoblastic differentiation markers, AP and Col I, by maximums of 66% (*P* < 0.03) and 145% (*P* < 0.001), respectively, compared with that in vehicle culture. The level of OC mRNA began to increase at 3 days and increased up to 147% by 6 days in a dose-dependent manner (*P* < 0.001; Fig. 3).

Protein secretion. As shown in Fig. 4, leptin significantly increased AP activity of hMS2-12 cells in a time (*P* = 0.02)- and

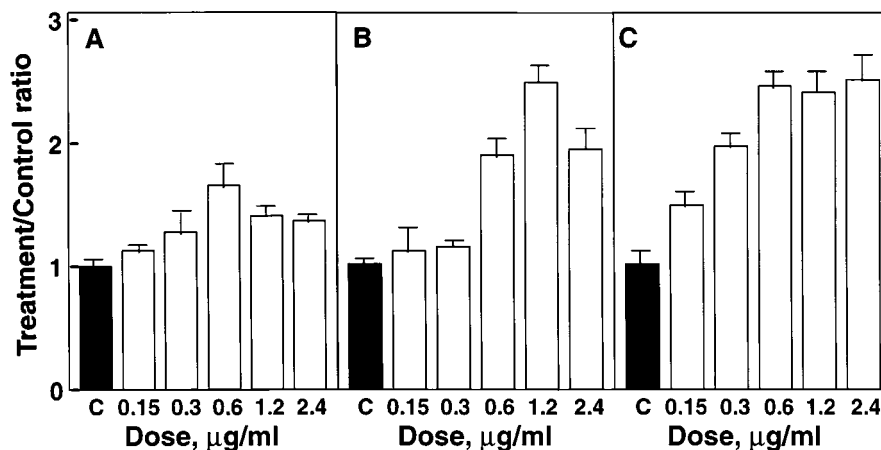


FIG. 3. Effect of leptin on osteoblastic marker mRNA expression. hMS2-12 cells were cultured in standard growth medium for 4 days at 34 C, and then cultured at 39.5 C in the standard differentiation medium in the presence of graded doses (0.15, 0.3, 0.6, 1.2, and 2.4 μ g/ml) of leptin (open bars) or vehicle (solid bar). Aliquots of cDNA synthesized from 2 μ g total RNA were amplified in a 25- μ l PCR reaction mixture with 0.25 μ l [α - 32 P]dCTP (10 μ Ci/ μ l) and corrected for GAPDH expression. Based on the maximal effect observed in the time-course studies, the dose effect of leptin was assessed at 3 days for AP and Col I mRNAs (A and B) and at 6 days for OC mRNA (C). Results are expressed as a percentage of the mean control values \pm SEM. The data are representative of a minimum of three separate experiments performed in triplicate. *P* < 0.05 for AP, and *P* < 0.001 for Col I or OC for differences from control, as assessed by multiple measures ANOVA.

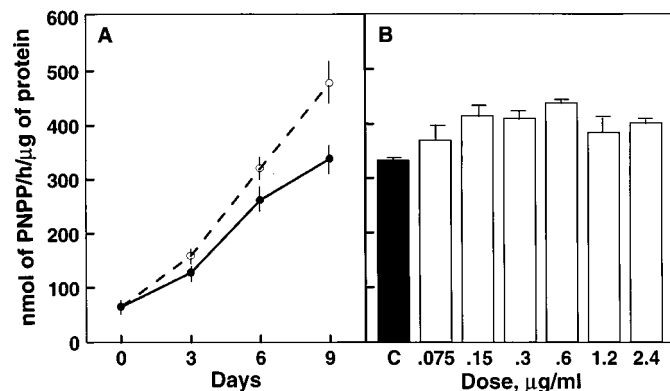


FIG. 4. Effect of leptin on AP activity. hMS2-12 cells were cultured in standard growth medium for 4 days at 34 C and then cultured at 39.5 C in the standard differentiation medium in the presence of leptin or vehicle. A, The cells were incubated in the standard differentiation medium at 39.5 C for 3, 6, and 9 days in the presence of 0.6 μ g/ml leptin (open circles) or vehicle (solid circles). B, The cells were incubated in the standard differentiation medium at 39.5 C for 3 days in the presence of graded dosages (0.075, 0.15, 0.3, 0.6, 1.2, and 2.4 μ g/ml) of leptin (open bars) or vehicle (solid bar). For both the dose- and time-response experiments, the AP activity was quantified as nanomoles of *p*-nitrophenylphosphate released per h/ μ g total cellular proteins. Results are expressed as the mean \pm SEM. The data are representative of three separate experiments performed in quadruplicate. $P = 0.02$ for time-dependent effects, and $P = 0.002$ for dose-dependent effects, as assessed by multiple measures ANOVA.

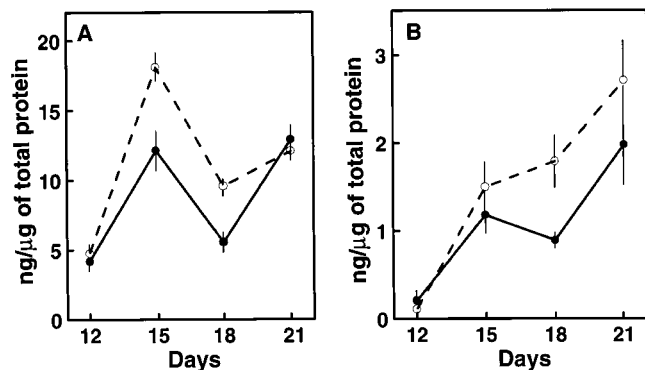


FIG. 5. Effect of leptin on type I collagen (A) and OC (B) secretion. hMS2-12 cells were cultured in standard growth medium for 4 days at 34 C, and then cultured at 39.5 C for 12–21 days in the standard differentiation medium in the presence of 0.6 μ g/ml leptin (open circles) or vehicle (solid circles). Type I collagen and OC values were normalized to micrograms of total cellular proteins. Results are expressed as the mean \pm SEM. The data shown are representative of three separate experiments performed in quadruplicate. $P < 0.01$, as assessed by multiple measures ANOVA for both Col I and OC.

dose ($P = 0.002$)-dependent manner, by 24% after 3 days in culture at 39.5 C and by 42% after 9 days, compared with the control value. Production of type I procollagen and OC proteins also increased significantly with time, by 72% ($P < 0.01$) and 37% ($P < 0.01$), respectively. Maximal osteocalcin production coincided with the onset of matrix mineralization (Fig. 5).

Mineralization of matrix. As assessed by Alizarin Red S staining, we observed a leptin-induced significant 59% increase in mineralization of the matrix in long term cultures ($P < 0.001$; Fig. 6).

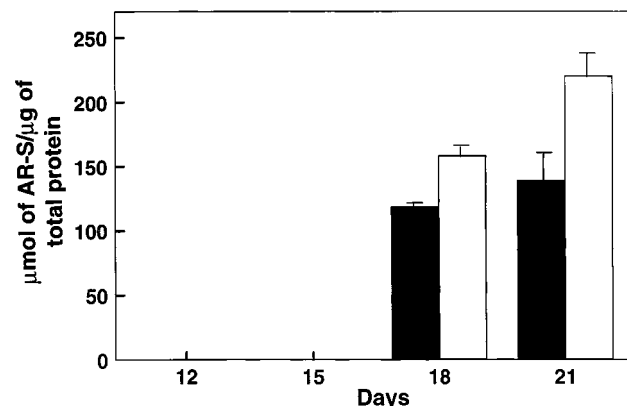


FIG. 6. Effect of leptin on mineralization of extracellular matrix. hMS2-12 cells were cultured in standard growth medium for 4 days at 34 C and then cultured at 39.5 C for 12–21 days in the standard differentiation medium in the presence of 0.6 μ g/ml leptin (open bars) or vehicle (solid bars). To quantify the formation of mineralized nodules, Alizarin Red S histochemical staining was performed. Alizarin Red S was then eluted from the matrix and measured by spectrophotometry at 540 nm. Results are expressed as the mean \pm SEM, in nanomoles of Alizarin red S per μ g total cellular proteins. The data shown are representative of three separate experiments performed in quadruplicate. No mineralization occurred before day 18. $P < 0.001$ compared with the corresponding control values, as determined by multiple measures ANOVA.

Effect of leptin on adipocyte differentiation

Expression of phenotype marker genes. Leptin decreased adipisin and leptin mRNA expression by 40% at 9 days ($P < 0.001$), which suggests that leptin decreases late adipocyte maturation (Fig. 7). Interestingly, gene expression of LPL, an early marker of adipocytic differentiation, was increased by leptin in a dose-dependent manner ($P < 0.001$) by 3 days (Fig. 7) and remained higher than control thereafter. PPAR γ_2 gene expression was nonsignificantly increased over the interval of 30 min to 72 h after leptin exposure (Fig. 8). No further changes were observed up to 9 days of treatment (data not shown).

Lipid droplet accumulation

Leptin decreased triglyceride accumulation in hMS2-12, as assessed by Oil Red O staining (Fig. 9). Whereas stained lipid droplets consistently appeared after 6 days at 39.5 C in both groups, leptin treatment progressively reduced lipid accumulation compared with the control value beginning after 9 days of leptin treatment ($P = 0.02$).

PPAR γ_2 protein production

We assessed possible differences in PPAR γ_2 protein content in cell lysates after leptin administration by Western blot analysis. Immunoblotting showed a 58-kDa band, consistent with the expected mol wt of PPAR γ_2 . Leptin did not change PPAR γ_2 protein levels after 1 and 3 days (Fig. 10).

Discussion

Although most of the interest in leptin has centered on its action on the hypothalamus (7), recent data show that leptin also acts on cells in peripheral tissues, including hematopoi-

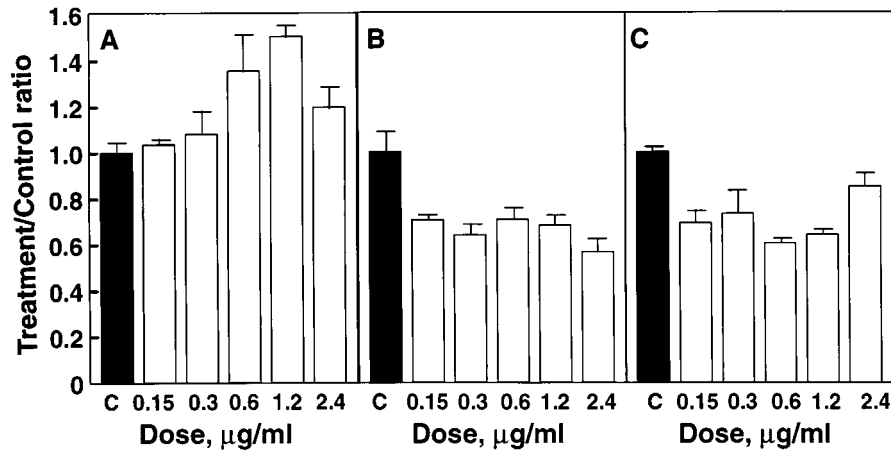


FIG. 7. Dose effect of leptin on adipocyte marker mRNA expression. hMS2-12 cells were cultured in standard growth medium for 4 days at 34 C and then cultured at 39.5 C in the standard differentiation medium in the presence of graded dosages (0.15, 0.3, 0.6, 1.2, and 2.4 µg/ml) of leptin (open bars) or vehicle (solid bar). Aliquots of cDNA synthesized from 2 µg total RNA were amplified in a 25-µl PCR reaction mixture with 0.25 µl [α - 32 P]dCTP (10 µCi/µl) and corrected for GAPDH expression. Based on the maximal effect observed in the time-course studies, the dose effect of leptin was assessed at 3 days for LPL mRNA (A) and at 9 days for adipin and leptin mRNA (B and C). Results are expressed as a percentage of the mean of the control value \pm SEM. The data are representative of a minimum of three separate experiments performed in triplicate. $P < 0.001$ for LPL, adipin, or leptin for differences from the control value, as assessed by multiple measures ANOVA.

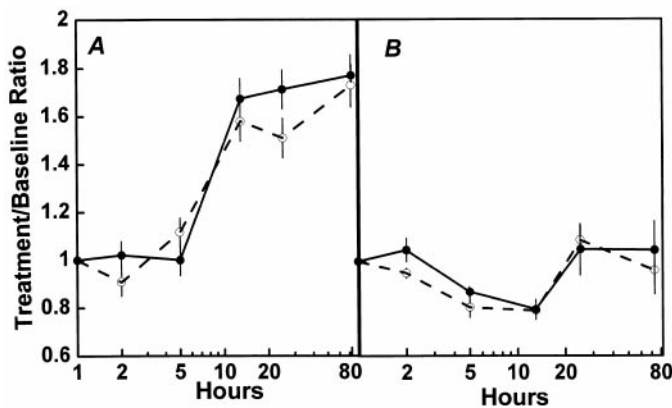


FIG. 8. Effect of leptin on Cbfa1 (A) and PPAR γ 2 (B) mRNA expression. hMS2-12 cells were cultured in standard growth medium for 4 days at 34 C and then cultured at 39.5 C in the standard differentiation medium in the presence of 0.6 µg/ml leptin (open circles) or vehicle (solid circles) for 30 min to 80 h. Aliquots of cDNA synthesized from 2 µg total RNA were amplified in a 25-µl PCR reaction mixture with 0.25 µl [α - 32 P]dCTP (10 µCi/µl) and corrected for GAPDH expression. Results are expressed as a percentage of the mean of the baseline values \pm SEM. The data are representative of three separate experiments performed in triplicate. No significant differences were observed between control and leptin treatments.

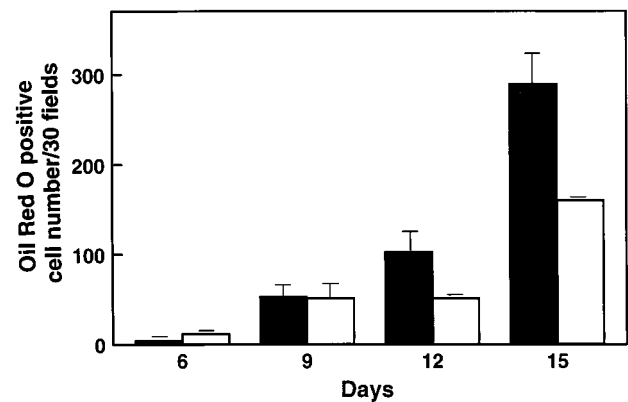


FIG. 9. Effect of leptin on cytoplasmic lipid droplet formation. hMS2-12 cells were cultured in standard growth medium for 4 days at 34 C, and then cultured at 39.5 C for 6–15 days in the standard differentiation medium in the presence of 0.6 µg/ml leptin (open bars) or vehicle (solid bars). To quantify the formation of cytoplasmic inclusions of neutral lipids, Oil Red O histochemical staining was performed. The percentage of Oil Red O-positive cells was determined by counting cells in 30 contiguous fields/well. Results are expressed as the mean \pm SEM. The data shown are representative of three separate experiments performed in quadruplicate. $P < 0.02$ compared with the corresponding control value, by multiple measures ANOVA.

etic precursor cells (12, 13), muscle cells (27), and adipocytes (10). All of these cells as well as osteoblasts (1) arise from a common precursor in bone marrow. This led us to hypothesize that leptin may affect the differentiation of osteoblast precursor cells. This hypothesis was tested using the hMS2-12 cell line that was established by immortalization of a stromal cell from normal human marrow. Under appropriate culture conditions, these cells are bipotential and can differentiate into either the osteoblast or adipocyte phenotype. Moreover, immortalization of hMS2-12 cells with the temperature-sensitive mutant of the SV40LTA is conditional. At the permissive temperature (when the SV40LTA is active), the cells can be rapidly expanded, whereas at the restrictive

temperature (when the SV40LTA is inactive), proliferation slows, and differentiation can be studied in what is essentially a clonal population of normal human marrow stromal cells (21, 24). Thus, the hMS2-12 cells are an ideal model system to study regulation of osteoblast differentiation.

We showed that the hMS2-12 cells were targets for leptin action by demonstrating that they expressed mRNA and protein for the leptin receptor. The short form of the receptor was present at both the permissive (34 C) and the restrictive (39.5 C) temperature, whereas the long splice variant was present in significant quantities only at the restrictive temperature. Gene expression for the different variants of OB-R increased when the cells were cultured at 39.5 C regardless

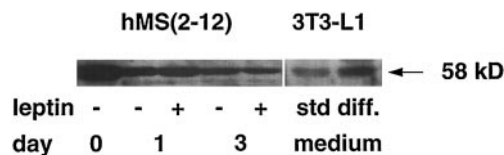


FIG. 10. Effect of leptin on PPAR γ_2 protein level. hMS2-12 cells were cultured for 4 days at 34 C and then incubated at 39.5 C in the presence of leptin (0.6 μ g/ml) or vehicle for 1 and 3 days. Equal aliquots of total cell lysates (50 μ g total protein) were examined on Western blots probed with a rabbit polyclonal IgG epitope affinity-purified anti-PPAR γ_2 antibody. Aliquots of the 3T3-L1 cell line cultured in standard or differentiation medium were used as a control. PPAR γ_2 protein was detected by chemiluminescence.

of the culture conditions (*i.e.* adipogenic, osteogenic, or both). Thus, alteration in OB-R expression appears to be induced by the occurrence of reduced proliferation and increased differentiation rather than the particular differentiation pathway. However, little is known about the regulation of long form OB-R expression and its specific activity. In fact, its presence has been reported in both early and lineage-restricted hematopoietic progenitors (12), in the placenta and in different fetal tissues (28), and in mature brown and white adipose tissues (29). Kellerer *et al.* could not detect the long form of OB-R in C2C12 myotubes, but leptin was able to activate Janus kinase-2- and insulin receptor substrate-2-dependent pathways in these cells (30). Only fasting has been recently reported to be associated with increased expression of mRNA for the long form of the OB-R in the hypothalamus (31). In our study, leptin administration had a modest inhibiting effect on the gene expression of its own receptor only in adipogenic conditions.

We were unable to demonstrate a significant effect of leptin on proliferation of hMS2-12 cells at either temperature. Leptin has been shown to exert a proliferative effect on hematopoietic progenitors (12, 13) and on pancreatic cells (32), but this does not appear to be the case for osteoblastic precursor cells. We cannot rule out that the absence or presence in low concentration of the long form of the OB-R might have contributed to this lack of proliferative effect at 34 C, as shown in the BaF3 hematopoietic cell line (33).

In contrast to the lack of an effect on proliferation, leptin clearly exerted a dose-dependent increase on osteoblast differentiation. These effects appear to be at the level of maturation rather than at the level of commitment. Cbfa1 is a recently discovered early response gene that is involved in commitment to the osteoblast differentiation pathway (22, 25, 26). We were unable to identify either early (hours) or late (days) effects of leptin on Cbfa1 gene expression. In contrast, there were consistent dose-dependent effects on steady state levels of mRNA and protein production of the osteoblast maturation markers AP, OC, and type I procollagen. Moreover, leptin treatment increased the mineralization of matrix, the hallmark of the osteoblast phenotype. The mechanism by which leptin increases osteoblastic differentiation is unclear. However, OB-R is closely related to the gp130 protein (34), and leptin binding to OB-R stimulates phosphorylation of the Jak/STAT kinase cascade, as do other gp130-dependent inducers of osteoblastic gene transcription, such as oncostatin M and leukemia inhibitory factor (35, 36).

The effects on adipocyte differentiation were more com-

plex. PPAR γ_2 is an early response gene that is involved in commitment to the adipocyte pathway (37). We failed to find a significant effect of leptin administration on mRNA expression or protein production of PPAR γ_2 . Steady state mRNA levels for LPL, a gene expressed early in the adipocyte differentiation pathway (38), were increased, whereas those for adiponin and leptin, genes that are expressed later during differentiation, were decreased. Most importantly, the accumulation of cytoplasmic lipid droplets, the hallmark of the adipocyte phenotype, was decreased by leptin, indicating that the overall effect of leptin was to decrease adipocyte differentiation. This decrease in neutral lipid accumulation is consistent with the findings of earlier studies showing that leptin lowers lipogenesis in the preadipocytic cell line 30A5 (10) and triglyceride accumulation in transfected rat pancreatic islets (11).

The reason for the paradoxical increase in expression of LPL despite the presence of an overall decrease in adipocyte differentiation is unclear. Interestingly, leptin administration to rodents increased LPL and decreased leptin gene expression in adipose tissue (29, 39). Also, overexpression of LPL in transgenic mice did not lead to an increase in fat deposition, but, rather, caused a decrease in the plasma triglyceride level (40). Thus, it is possible that induction of LPL may provide energy for marrow stromal cell differentiation by enhancing the hydrolysis of extracellular triglycerides for cells early in either the adipocyte or osteoblast pathway.

The effects of leptin on marrow stromal cell osteoblastic differentiation differ from those of bone morphogenetic protein-2 in this model system (Gori, F., *et al.*; manuscript submitted for publication). First, the effect of leptin on osteoblast differentiation was less pronounced quantitatively. Second, the primary effect of bone morphogenetic protein-2 appears to be on commitment to the osteoblast pathway through an early increase in Cbfa1 gene expression, whereas leptin did not affect Cbfa1 expression. Thus, leptin appears to primarily act at the level of osteoblast maturation, rather than at the level of commitment. Because a recent study found that human bone marrow adipocytes in primary culture had high leptin expression (41), leptin could serve as an autocrine/paracrine factor to modulate the differentiation of marrow stromal cells as well as hematopoietic precursor cells (12, 13) in addition to its well established endocrine role.

Our observations may be relevant to the clinical observations that obesity is associated with increased bone mineral density (14, 42–44) and that increased body mass index protects against postmenopausal bone loss (45, 46). These associations have generally been attributed to the mechanical effect of increased load bearing on increasing bone formation and to the effect of higher circulating estrogen levels associated with increased aromatase activity in the larger mass of adipose tissue (19). However, fat mass and bone mineral density (BMD) are still directly and strongly correlated after adjusting for differences in serum estrogen levels (47, 48). Moreover, fat mass (49) or body weight (50) and BMD are correlated in women independent of menopausal status. As obesity is also associated with higher circulating leptin levels (20), it is possible that the effect of leptin on enhanced maturation of marrow precursor cells into osteoblasts is a major

factor in mediating the relationship between fat mass and BMD. Indeed, a recent clinical study demonstrated a direct relationship between serum leptin level and total body bone area in pubertal girls (51).

In summary, we report here a direct osteogenic effect of leptin on a human marrow stromal cell line with the capability to differentiate to either osteoblasts or adipocytes. We found that leptin enhances osteoblastic differentiation and inhibits adipocytic differentiation. Leptin appears to act by enhancing the entire osteoblast maturation pathway and by inhibiting the late adipocytic maturation pathway, rather than by acting at the level of commitment to either pathway. Thus, leptin could serve as a previously unrecognized physiological regulator of the balance between the fat and bone compartments. The possibility that leptin could be useful as a therapeutic agent for the treatment of osteoporosis deserves evaluation.

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New Roles for Multifunctional Peptides

Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating peptide (PACAP) are well-established as agents in the flow of blood to the intestines. But new research shows important additional roles for these and related peptides, including: their affect on glucose production as it relates to the pathology of diabetes; their influence on nerve growth and nerve regeneration; PACAP's affect on appetite and its possible aide in the development of anti-obesity drugs; and VIP's function as a major player in the ability of the intestines to rebuild themselves following irradiation in cancer treatment. These and other findings are considered in VIP, PACAP, and Related Peptides: Third International Symposium, volume 865 of the *Annals of the New York Academy of Sciences*.

The volume is edited by Wolf-Georg Forssmann, Lower Saxony Institute for Peptide Research in Hannover, Germany, and Sauni I. Said, State University of New York at Stony Brook and the Veterans Affairs Medical Center, Northport, New York. The eight sections of the book are: Regulation of Gene Expression and Processing of VIP-PACAP Peptides; Biochemistry of VIP-PACAP-Related Peptide Receptors; Signal Transduction and Intracellular Metabolisms Induced by VIP and PACAP; Physiology of Effector Mechanisms; Neurobiology of VIP-PACAP-Related Peptides; Pharmacology of VIP-PACAP Agonists and Antagonists; Progress in Clinical Research of VIP-PACAP Peptides; and Poster Papers. Included are 40 papers and 48 poster papers presented at a 1997 conference convened in Freiburg, Germany, December 1998/611 pages/\$140.00/ISBN: 1-57331-153-7 (cloth), 1-57331-154-5 (paper).