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Leptin Acts as a Growth Factor on the Chondrocytes of Skeletal Growth Centers

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ABSTRACT

Childhood obesity frequently is associated with an increase in height velocity and acceleration of epiphyseal growth plate maturation despite low levels of serum growth hormone (GH). In addition, obesity is associated with higher circulating levels of leptin, a 16-kDa protein that is secreted from the adipocytes. In this study, we evaluated the direct effect of leptin on the chondrocyte population of the skeletal growth centers in the mouse mandibular condyle, a model of endochondral ossification. We found that chondrocytes in the growth centers contain specific binding sites for leptin. Leptin, at a concentration of 0.5–1.0 μg/ml, stimulated in a dose-dependent manner the width of the chondroprogenitor zone (up to 64%), whereas higher concentrations had an inhibitory effect. Leptin induction of both proliferation and differentiation activities in the mandibular condyle was confirmed by our findings of an increase in bromodeoxyuridine (BrdU) incorporation into DNA and in (acidic) Alcian blue (AB) staining of the cartilaginous matrix. Leptin also increased the abundance of the insulin-like growth factor (IGF) I receptor and IGF-I receptor messenger RNA (mRNA) within the chondrocytes and the progenitor cell population. Our results indicate that leptin acts as a skeletal growth factor with a direct peripheral effect on skeletal growth centers. Some of its effects on the growing bone may be mediated by the IGF system via regulation of IGF-I receptor expression. We speculate that the high circulating levels of leptin in obese children might contribute to their growth. (J Bone Miner Res 2002;17:1034–1043)

Key words: leptin, insulin-like growth factor I, insulin-like growth factor I receptor, epiphyseal growth plate, endochondral ossification

INTRODUCTION

CHILDREN'S GROWTH is dependent on an intact and functioning growth hormone (GH)—insulin-like growth factor (IGF) I) axis. However, there are some clinical conditions in which normal growth persists in the presence of low GH serum levels. In 1962, Matson[1] was the first to describe normal height velocity in children after craniopharyngioma surgery in association with marked obesity, and similar findings in larger series have been reported since then.[2–7] Children with exogenous obesity also usually show an increase in height velocity concomitant with acceleration of bone epiphyseal maturation,[8] despite low plasma GH levels even during the phase of acceleration. The decrease in GH in these cases has been attributed to both decreased secretion by the pituitary gland and increased clearance from the plasma.[9,10] The mechanism whereby obese children continue to grow despite low levels of GH has not been elucidated fully.

Circulating leptin levels are increased in obesity and correlate positively with fat mass.[11] This is true for both adults and children.[12] Studies using the ob/ob mouse

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model have shown that leptin is the product of the ob gene, which when mutated, results in obesity.[13] Leptin, a 16-kDa protein is secreted mainly by the white adipose tissue and regulates food intake and body weight by negative feedback at the hypothalamic nuclei.[14] The biological activity of leptin is mediated by specific receptors that belong to class I cytokine receptors, which are single membrane-spanning receptors. Leptin receptors were first cloned by Tartaglia[15] who showed that they exist in several variants because of alternative splicing. In the hypothalamus, the predominant and the only biologically active isoform of leptin receptor is the long receptor.[16] Leptin receptors also have been identified in various peripheral tissues mediating a variety of activities.[16] Despite the fact that the central activity of leptin differs between man and mice, the peripheral effect of leptin in both species has yet to be explored fully.

In this study, we report the presence of leptin receptors in cartilaginous skeletal growth centers of a young mouse involved in leptin-induced skeletal growth. We evaluated the direct effect of leptin on the chondrocyte population of skeletal growth centers in the mice mandibular condyle, a model of endochondral ossification.

MATERIALS AND METHODS

Culture system

Mandibular condyles from 6-day-old Institute of Cancer Research (ICR) mice were grown in an organ culture as previously described.[17,18] The BGJ_b-Fitton Jackson Modification (Beth Haemek, Israel) was supplemented with 2% fetal calf serum (FCS), 100 μg/ml of ascorbic acid, and antibiotics. The tissues were incubated for 2–4 days at 37°C, 5% CO_2 /95% air, and maximal humidity in the presence of 0.5, 1.0, or 1.5 μg/ml of ovine leptin or under control conditions. For the bromodeoxyuridine (BrdU) incorporation assay, cultures were treated with 100 μM of BrdU for the last 18 h of incubation.

For IGF-I immunoinhibition, condyles were incubated in the presence of anti–IGF-I antibodies, diluted 1/500 in the culture medium. At the end of the incubation period, the explants were washed thoroughly with cold Hanks' buffer, fixed with neutral-buffered formalin, and routinely processed for paraffin embedding.

Alcian blue + hematoxylin and eosin staining

Paraffin sections (6 μm) were deparaffinized in xylene, hydrated in graduated ethanols, and pretreated with 3% acetic acid for 3 minutes. Then, sections were stained with 1% Alcian blue (AB) at pH 2.5 for 30 minutes, thoroughly rinsed with tap water, and counterstained with hematoxylin and eosin (H&E).

Morphometric studies

AB + H&E–stained sections were used in the morphometric studies. The length of the various cellular layers and the number of $BrdU^+$ nuclei and the relative area occupied by them were determined with an Olympus Cue-2 image analysis system with appropriate morphometry software (Olympus Corp., Lake Success, NY, USA). The system consists of a Zeiss Universal R photomicroscope (×10 objective; Zeiss, Oberkochen, Germany) fitted with a Panasonic WV-CD50 camera (Matsushita, Mississauga, Ontario, Canada). The video image is viewed on a Sony 14-in color monitor attached to an IBM-compatible PC.

BrdU incorporation into DNA

Condyles cultured under either normal or leptin-treated conditions were incubated with BrdU for the last 18 h of incubation (as described previously). At the end of the incubation period, the condyles were washed thoroughly with Hanks' buffer and processed routinely for paraffin embedding. Detection of BrdU-labeled cells was performed on deparaffinized 6-μm-thick sections using the Zymed BrdU staining kit (catalogue no. 93–3943; Zymed Laboratories, Inc., San Francisco, CA, USA) according to the manufacturer's instructions. Briefly, after quenching the endogenous peroxidase, sections were mildly trypsinized and then reacted with anti-BrdU antibody (Ab) after biotinylated second Ab with streptavidin-peroxidase–conjugate and diaminobenzidine (DAB) as a substrate; counterstaining was performed with hematoxylin.

Immunohistochemistry

Deparaffinized paraffin sections were reacted for 2 h at room temperature with one of the following specific antibodies: anti-leptin receptor monoclonal Ab (MAb; catalogue no. sc-8391; Santa Cruz Biotechnology, Santa Cruz, CA, USA; this Ab is mapping the protein between the aa 877–894), rabbit anti–IGF-I receptor (anti–α-subunit; catalogue no. SC-712; Santa-Cruz Biotechnology), mouse anti-cartilage proteoglycans (MAB 2010; Chemicon International, Inc., Temecula, CA, USA), mouse anti-collagen type II (catalogue no. MAB 8887; Santa-Cruz Biotechnology), or mouse antiproliferating cell nuclear antigen (PCNA; catalogue no. 08–0110; Zymed Laboratories, Inc.). This was followed by incubation with an appropriate biotinylated second Ab, with streptavidin-peroxidase conjugate, and aminoethyl carbazole (AEC) as a substrate (Histostain-SP kit; Zymed Laboratories, Inc.); counterstaining was done with hematoxylin.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western analysis

Tissue lysates prepared from pools of 3-day cultured mandibular condyles treated with 0.5 μg/ml of leptin, nontreated, and from murine brains were separated by reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membrane. Blots were incubated with anti-leptin receptor MAb's or rabbit ant–IGF-I receptors Ab's and detected by rabbit anti–mouse-horseradish peroxidase (HRP; Sigma-Aldrich, St. Louis, MO, USA) and goat anti–rabbit-HRP conjugates (Upstate Biotechnology, Lake Placid, NY, USA) and re-

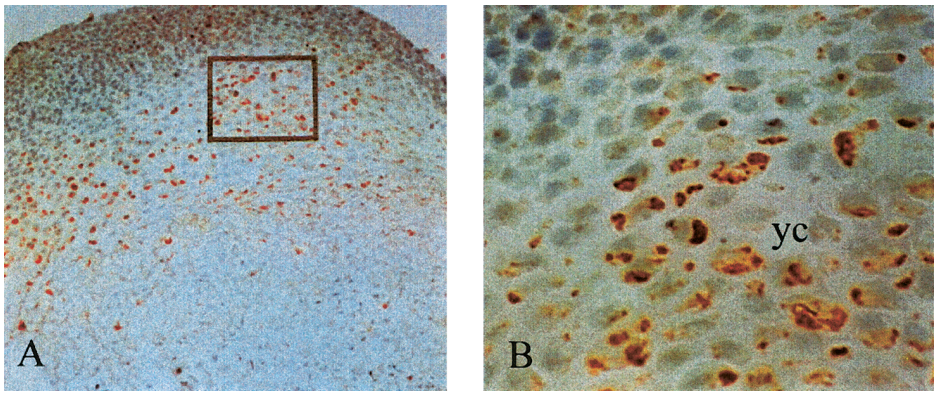

FIG. 1. Immunohistochemical localization of the leptin receptor within the 6-day-old murine-derived skeletal growth centers. Deparaffinized sections of mandibular condyle were reacted with rabbit anti-leptin receptor and specific binding was detected with the avidin-biotin-peroxidase system. (A) Leptin receptors were localized in the chondrocytic population of the mandibular condyle (magnification ×190). (B) Higher magnification revealed that leptin receptors were especially located in the chondrocyte (yc) population of the mandibular condyle (magnification ×240).

vealed by chemiluminescence reagent (Perkin Elmer, Shelton, CA, USA).

In situ hybridization

For this study, 6-μm paraffin sections were loaded on precleaned poly-L-lysine–coated slides, deparaffinized with xylene, hydrated with graduated ethanols, and treated with 3% H_2O_2 in methanol to neutralize endogenous peroxidase. Sections then were treated for 15 minutes with 12.5 μg/ml of proteinase K, rinsed with 2 mg/ml of glycine, and acetylated in 0.5% acetic anhydride in 0.1 M of Tris at pH 8.0. Thereafter, sections were postfixed with 4% paraformaldehyde/phosphate buffered saline (PBS); prehybridization for 10′ in 2× SSC was followed by 1 h in hybridization buffer: 50% formamide, 0.5 mg/ml of salmon sperm DNA, 4× SSC, and 1× Denhardt. Hybridization was done overnight (18 h) at 42°C and maximal humidity was achieved with a 5-ng/μl digoxigenin (DIG)-labeled probe (see the following section). At the end of the incubation period, slides were rinsed in SSC under increasingly stringent conditions and then with 0.1 M of Tris and 0.15 M of NaCl at pH 7.5. Hybrids were detected using anti-DIG Ab's conjugated with peroxidase (Roche Diagnostics GmbH, Mannheim, Germany) and AEC as a substrate, and counterstained with hematoxylin.

DIG-labeled antisense RNA probes for in situ hybridization

We used probes for mouse IGF-I receptor (IGF-IR) cloned in pBluescript SK^+ amp^+, for mouse IGF-I cloned in pGEM3 amp^+ (386 base pairs [bp]). After linearization, antisense RNA was transcribed with the (Sp6/T7) DIG-RNA labeling kit (Boehringer Mannheim) according to the manufacturer's instructions.

Statistics

Each point in the morphometric studies represents a mean of measurements performed in three to five different experiments with at least three condyles cultured under each culture condition in each experiment and at least three measurements of each condyle. Results are shown as mean ± SD. Statistical significance was determined with analysis of variance (ANOVA) followed by Bonferroni pairwise *t*-test analysis.

RESULTS

Mandibular condyle contains leptin receptors

Mandibular condyles derived from 6-day-old ICR mice were fixed and routinely processed for paraffin embedding as described previously. Deparaffinized sections were reacted with anti-leptin receptor Ab and positive staining was detected using relevant biotinylated second Ab (Zymed detection kit) as described previously. Staining was positive as shown in Fig. 1. The mandibular condyle contains specific receptors for leptin (Fig. 1A), and these are especially abundant in the chondrocytes (Fig. 1B) but also are present in the hypertrophic cells.

Mandibular chondrocytes express heavy-type isoform of leptin receptors

Lysates derived from 6-day-old mandibular condyles treated and nontreated with 0.5 μg/ml of leptin as well as from murine brain were separated on SDS-PAGE and electrotransferred onto nitrocellulose membrane. Western analysis with anti-leptin receptor Ab (Fig. 2A) reveals a major band that corresponds to 148 kDa and a minor one of ~80 kDa. Both are identical to those appearing in brain lysates. Leptin (1.0 μg/ml) up-regulates the levels of its own receptors; however, higher concentrations (1.5 μg/ml) of leptin do not further increase the level of leptin receptors. Densitometry has shown that brain tissue contains 130% more leptin receptors than does the mandibular condyle. This probably is caused by the low cellularity of the cartilage tissue in comparison to the brain.

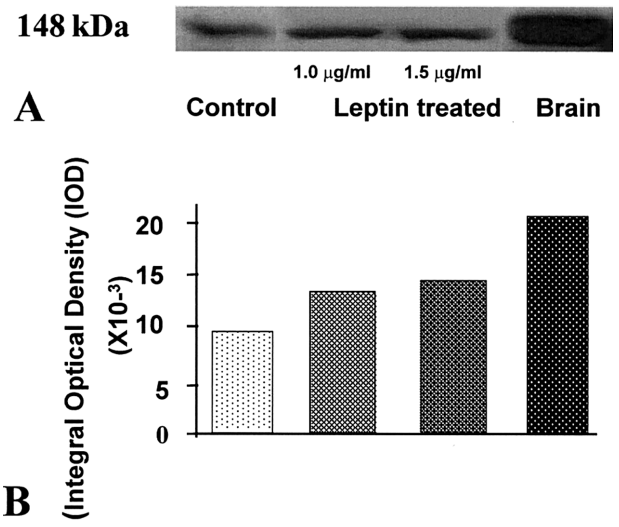

FIG. 2. SDS-PAGE analysis of leptin receptors in control mandibular chondrocytes, treated with 1.0 μg/ml of leptin, 1.5 μg/ml of leptin, and brain tissue, blots were incubated with anti-leptin receptors MAb's that recognize the intracellular fragment, aa 877–894. (A) The 148-kDa isoform appears in both the brain and the mandibular condyle. Densitometry (B) reveals that the level of leptin receptors in the brain is 130% higher than that of the condyle. The 1.0-μg/ml of leptin slightly increases the level of leptin receptors.

Leptin stimulates proliferation activity in the cultured mandibular condyle

Mandibular condyles were cultured in the presence of various concentrations of leptin (0.25, 0.5, 1, or 1.5 μg/ml) for several days with the addition of BrdU for the last 18 h of incubation. At the end of the incubation period, condyles were processed routinely for paraffin embedding and deparaffinized sections were detected for BrdU-containing DNA using the Zymed BrdU detection kit (based on specific anti-BrdU Ab).

As shown in Fig. 3, the level of BrdU–containing nuclei increased markedly over the untreated control after 2 days of incubation starting with 0.5 μg/ml of leptin (Fig. 3C vs. Fig. 3A). A lower dose of leptin (-0.25 μg/ml) was still ineffective in stimulating proliferating activity (Fig. 3B).

Morphometric analysis revealed a 125% increase of the $BrdU^+$ cells; this figure rose by an additional 34% with 1.0 μg/ml of leptin. However, in the presence of 1.5 μg/ml of leptin, the $BrdU^+$ cells increased by only 15% over the control (Fig. 3D). This increase in proliferation activity was reflected by the enhancement of the chondroprogenitor zone. We applied ANOVA to the four groups (control group and the three different leptin doses) and found that the difference between the groups is highly significant ($p < 0.001$). Bonferroni analysis of paired *t*-tests reveals significant differences between control and 0.5 μg/ml of leptin ($p < 0.001$) and between control and 1.0 μg/ml of leptin ($p < 0.001$). No significant difference was found between the control and the 1.5-μg/ml leptin-treated group. Figure 3E shows the results of a dose-response study performed on 4-day leptin-treated cultures. Leptin at a concentration of 0.5–1.0 μg/ml had a dose-dependent stimulatory effect on the size of the proliferative zone of the condyle (17% and 23% increase over the control, respectively). ANOVA analysis has shown that the difference between the groups is highly significant ($p < 0/001$). The stimulatory effect of leptin was significantly reduced by 1.5 μg/ml of leptin ($p < 0/001$ compared with 0.5 μg/ml or 1.0 μg/ml of leptin, pairwise comparison according to Bonferroni).

Leptin stimulates the differentiation of chondrocytes within the mandibular condyle

Normal skeletal growth depends on the coupling of proliferation and differentiation activities within the skeletal growth centers. The effect of leptin on differentiation was studied with acidic (pH 2.8) AB staining of the cartilage, which indicates a highly sulfated proteoglycan-containing matrix. Figures 4A and 4B depict the increase in AB staining after a 4-day culture in the presence of 1.0 μg/ml of leptin (note the positive staining within the chondroprogenitor zone in Fig. 4B).

Thereafter, using a more direct approach, we examined the production of chondroitin sulfate by these cells. The chondroitin sulfate was localized by immunohistochemistry staining using specific anticartilage proteoglycan Ab's. As shown in Fig. 5, 1.0 μg/ml of leptin (Fig. 5B) significantly increased the content of chondroitin sulfate within the chondrocytes compared with that of control (Fig. 5A), whereas 1.5 μg/ml had only a mild stimulatory effect (Fig. 5C). Parallel sections reacted with normal rabbit serum served as a negative control (data not shown).

Leptin increases the overall size of the mandibular condyle

The effect of leptin on the condylar growth was estimated morphometrically. Condyles cultured for 4 days in the presence of either 0.5 μg/ml or 1.0 μg/ml leptin were processed routinely for paraffin embedding, and H&E-stained sections were used for morphometric analysis. Results shown in Fig. 6 reveal that treatment with 0.5 μg/ml leptin for 4 days increases the overall size of the condyle by 8% ($p < 0.05$). However, the 1.5-μg/ml of leptin decreases significantly ($p < 0.001$) the overall condylar size.

Leptin increases IGF-I-receptors in the cultured condyle

One of the most important local regulatory factors in the development of endochondral ossification is IGF-I. We studied the effect of leptin on the expression of IGF-I receptors at both the protein and the messenger RNA (mRNA) levels using immunohistochemical and in situ hybridization techniques, respectively. Results are depicted in Fig. 7. After 3 days of incubation, 1.0 μg/ml of leptin (Fig. 7B) markedly increased the production of IGF-I receptors in the entire chondrocytic population as compared with the control (Fig. 7A); 1.5 μg/ml had only a mild stimulatory effect (Fig. 7C). In situ hybridization, using a specific DIG-labeled riboprobe for IGF-I receptor yielded

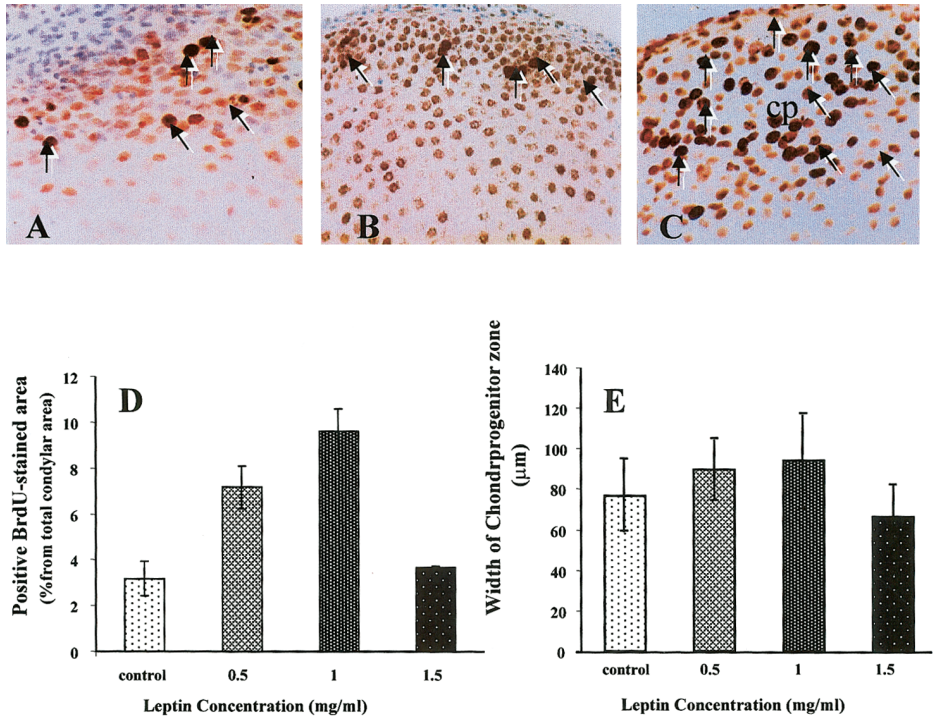

FIG. 3. The effect of different leptin concentrations on cell proliferation of cultured mandibular condyles. Condyles were incubated alone or in the presence of 0.25, 0.5, 1.0, or 1.5 μg/ml of leptin for 2 days. BrdU (100 μM) was added for the last 18 h of incubation. Sections were studied for the presence of BrdU-labeled nuclei using anti-BrdU Ab's. ANOVA analysis has shown that the difference between the groups is highly significant ($p < 0.001$). (A) Compared with controls, incubation in the presence of 0.5 μg/ml of leptin for 2 days markedly increased the BrdU-containing cell population (C) within the proliferating zone (cp); (B) 0.25 μg/ml is ineffective (magnification $\times$240). (D) Morphometric analysis showed a 125% ($p < 0.001$ pairwise comparison according to Bonferroni) and 159% ($p < 0.001$, pairwise comparison according to Bonferroni) increase in the relative $BrdU^+$ area induced by 0.5 μg/ml and 1.0 μg/ml of leptin, respectively. Leptin, in a dose of 1.5 μg/ml, was not significantly different from that in the control. (E) Morphometric studies performed on condyles after 4 days of culture showed that 0.5 μg/ml and 1.0 μg/ml of leptin induced an increase of 17% and 23% in the width of the chondroprogenitor zone. The stimulatory effect of leptin was significantly reduced by 1.5 μg/ml of leptin ($p < 0.001$ compared with 0.5 μg/ml or 1.0 μg/ml leptin; ANOVA).

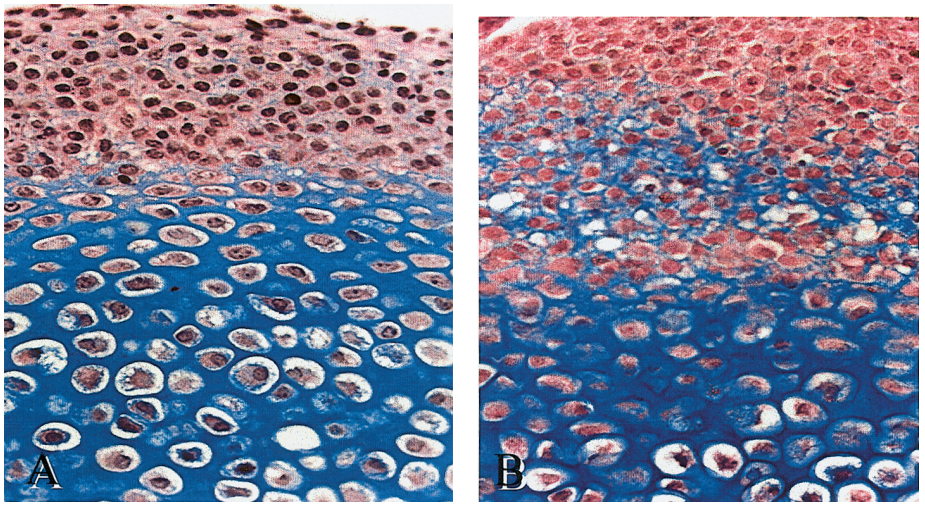

FIG. 4. Effect of leptin on metachromatic staining of the cartilaginous matrix of the mandibular condyle. Condyles cultured for (A) 3 days alone or (B) in the presence of 1.0 μg/ml of leptin were stained in acidic (pH 2.5) AB. Leptin treatment increased both the metachromatic-stained area and the intensity of the staining (magnification $\times$240).

positive-stained cells (red) distributed among the chondroprogenitor and chondrocytic cells of the 1.0-μg/ml leptin-treated condyle (Fig. 7E) but only a very faint positive reaction in the control (Fig. 7D). Again, the higher leptin dose (1.5 μg/ml; Fig. 7F) was less potent. This indicates that leptin induced a dose-dependent up-regulation of IGF-I receptors in the chondrocytic population of the mandibular condyle is at the level of gene regulation. Parallel sections

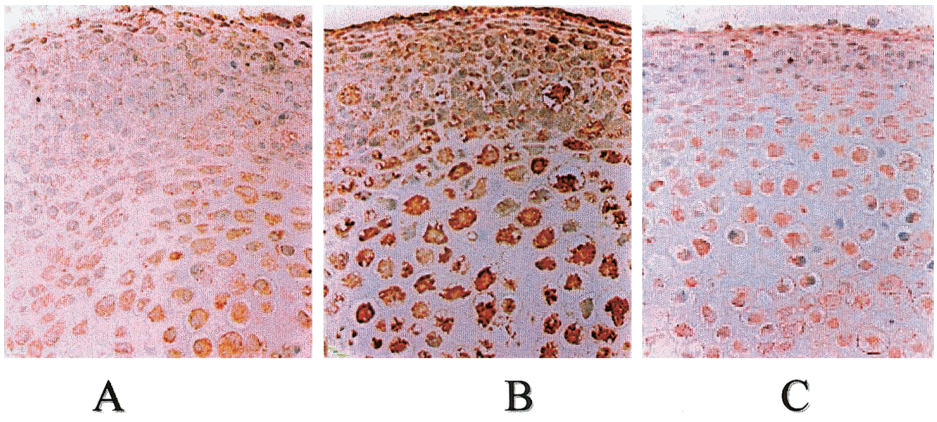

FIG. 5. Synthesis of chondroitin sulfate in the leptin-treated mandibular condyles. Chondroitin sulfate was localized in (A) untreated, (B) 1.0 μg/ml of leptin-treated, or (C) 1.5 μg/ml of leptin-treated 3-day cultured condyles using specific antichondroitin sulfate MAb. The positive reaction appears in red; nuclei are counterstained with hematoxylin blue (magnification $\times$240).

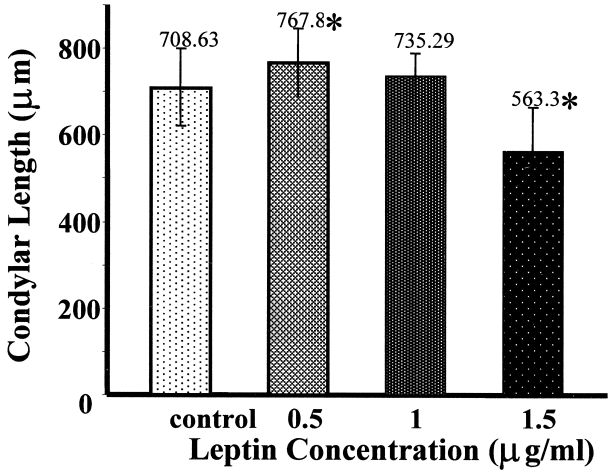

FIG. 6. The effect of leptin on the overall condylar growth. Morphometry of H&E-stained sections derived from 4-day cultured condyles treated with 0.5, 1.0, or 1.5 μg/ml of leptin or nontreated. ANOVA analysis has shown that the difference between the four groups is highly significant ($p < 0.001$); 0.5 μg/ml of leptin increased the overall length of the condyle by 8%; 1.5 μg/ml of leptin decreased significantly ($p < 0.001$; ANOVA) the overall condylar size.

incubated with DIG-labeled sense IGF-I receptor riboprobe served as a negative control (data not shown).

Quantification of leptin's effect on the IGF-I receptors levels (Fig. 8) was estimated by Western analysis of tissue lysates derived from 3-day cultures of control, 0.5 μg/ml and 1.0 μg/ml leptin-treated condyles (Fig. 8 A). Densitometry of the bands (Fig. 8B) reveals an increase of 63% and 79% by 0.5 μg/ml and 1.0 μg/ml leptin, respectively.

Time-course effect of leptin on the levels of IGF-I receptors

Because IGF-I is considered to be a regulator of early endochondral ossification functions such as proliferation and differentiation, we examined the effect of leptin on the changes in the levels of IGF-I receptors throughout the culture period. Cultures were incubated for 2, 3, and 4 days in the presence or absence of 1.0 μg/ml of leptin. Levels of IGF-I receptor were assessed immunohistochemically. Results, shown in Fig. 8, reveal that levels of IGF-I receptors are already increased after 2 days of leptin treatment (Fig. 9B) as compared with the untreated control (Fig. 9A). Maximal stimulatory effect appears on the third day of treatment (Fig. 9C); after 4 days of treatment (Fig. 9D), the level of IGF-I receptors is similar to that of the control.

Immunoinhibition of IGF-I blunted the stimulative activity of leptin on the condylar growth

To verify the foregoing assumption that leptin-induced condylar growth is mediated by IGF-I system, we studied the effect of immunoneutralization of IGF-I on leptin activity. Cultures were incubated for 3 days in the presence of anti–IGF-I Ab diluted 1/500 with or without 0.5–1.0 μg/ml of leptin. The effects of leptin in the presence of IGF-I immunoinhibition on proliferation and differentiation activities are shown in Figs. 10 and 11, respectively.

Proliferation was studied by detection of the expression of PCNA immunohistochemically. Results shown in Fig. 10 depict that the inductive effects of 1.0 μg/ml of leptin (Fig. 10B) in comparison with the untreated control (Fig. 10A) are blunted completely in the presence of anti–IGF-I (Fig. 10C). Figure 11 shows the effects of leptin on collagen type II synthesis in the presence (Fig. 11C) and absence (Fig. 11B) of anti–IGF-I antibodies. Leptin seems to increase collagen type II expression within the chondroblastic zone (cb, Fig. 11B) as compared with the control culture (Fig. 11A). Immunoinhibition of IGF-I (Fig. 11C) decreases collagen type II secretion within the chondroblastic zone but left some positive reaction within the hypertrophic zone.

DISCUSSION

Leptin, a small polypeptide hormone secreted primarily by the adipocytes, controls body weight after its binding to a specific receptor located in the hypothalamus. However, 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.[19] Leptin has

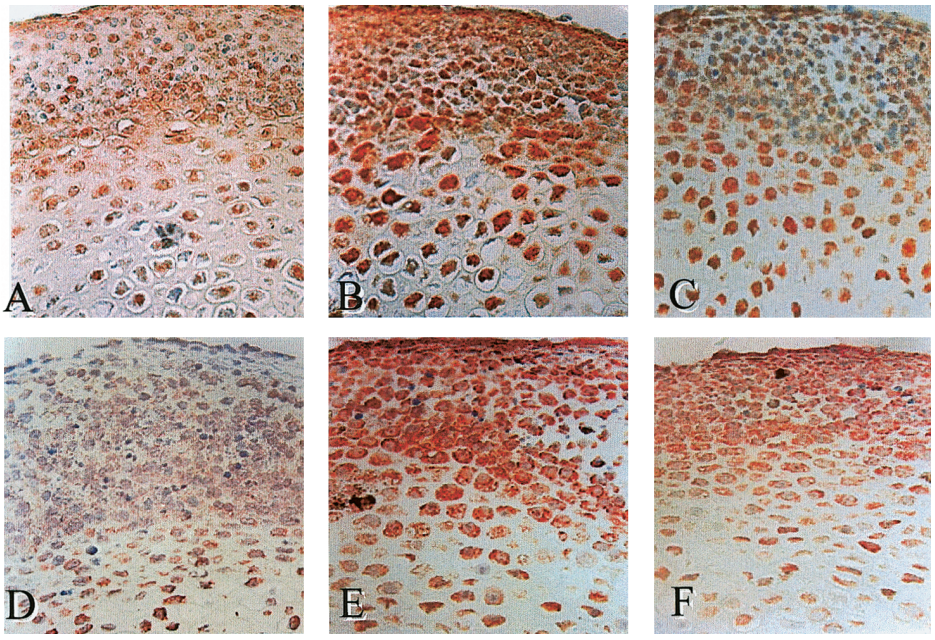

FIG. 7. Leptin's effect on the IGF-I receptor and IGF-I receptor mRNA abundance in the mandibular condyle. Condyles cultured (A and D) under control conditions, (B and E) with 1.0 μg/ml of leptin, or (C and F) with 1.5μg/ml of leptin for 3 days were paraffin embedded. (A–C) Deparaffinized sections were assayed for the IGF-I receptor abundance by immunohistochemistry using specific rabbit anti–IGF-I receptor Ab. The positive cells are stained red (arrows). (D–F) In situ hybridization technique, using DIG-labeled antisense probe for IGF-I receptors was used for assessing the IGF-I receptor mRNA abundance. The positive cells are stained in purple (arrows).

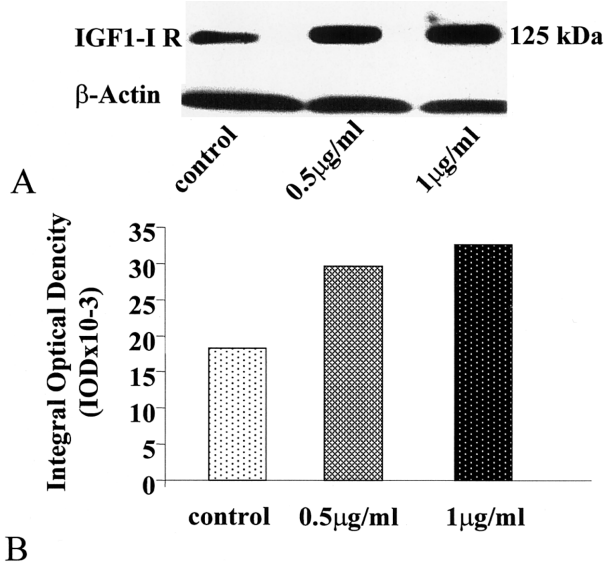

FIG. 8. Quantification of the effect of leptin on the levels of IGF-I receptors in the mandibular condyle. Densitometry (B) of western analysis (A) of 3-day-old control and 0.5 μg/ml or 1.0 μg/ml of leptin-treated cultures reveals an increase of 63% and 79% by 0.5 μg/ml and 1.0 μg/ml of leptin, respectively.

been reported to act on hematopoietic precursor cells,[20,21] adipocytes,[22] cultured hepatocytes,[23] and pancreatic islet cells[24] and on human marrow stromal cells enhancing osteoblast differentiation and inhibiting adipocyte differentiation.[25]

In this study, we show that chondrocytes in the skeletal growth centers contain specific binding sites for leptin. We found a 148-kDa isoform of leptin receptor in the chondrocytes, which also appeared in the brain. We still do not know whether this isoform is identical to the long leptin receptor b (ObR-b) isoform of the brain. However, it has been shown that several peripheral activities of leptin probably are not mediated by the long form of the leptin receptor[26] principal found in the brain. We have also found that leptin stimulates, in a dose-dependent manner, the width of the proliferating zone. The increment in BrdU incorporation into DNA and the increase in the expression of chondroitin sulfate within the cartilaginous matrix confirms that leptin induces both proliferation and differentiation activities in the mandibular condyle, which is reflected ultimately in an increase in the overall size of the treated condyle.

Leptin concentrations used in this study are rather high (0.5–1.5 μg/ml) in comparison with physiological levels. Nonetheless, this range of concentrations is quite common in other in vitro studies,[25,27,28] probably because of the fact that part of the leptin activity is blocked by the serum component of the medium used in in vitro studies. Using this range of doses, we found a dose-dependent effect of leptin on the condylar growth. In most cases, 0.5 μg/ml and 1.0 μg/ml of leptin were stimulatory and 1.5 μg/ml was less effective. Because no down-regulation of leptin receptors has been observed in the presence of 1.5 μg/ml of leptin, it seems more likely that the reduction of the stimulatory effect under higher doses of leptin probably is caused by cross-reactivity of leptin with other cytokine type I receptors that have catabolic effects on the condylar development, for example, interleukin (IL)-6.

Childhood obesity is associated with accelerated growth. Childhood growth usually is dependent on an intact and functioning GH–IGF-I axis. However, measurements performed both during sleep and over 24 h have shown that GH secretion in children with obesity is reduced under physiological conditions.[29,30] In addition, obese individuals are characterized by abnormally low GH responses to pharma-

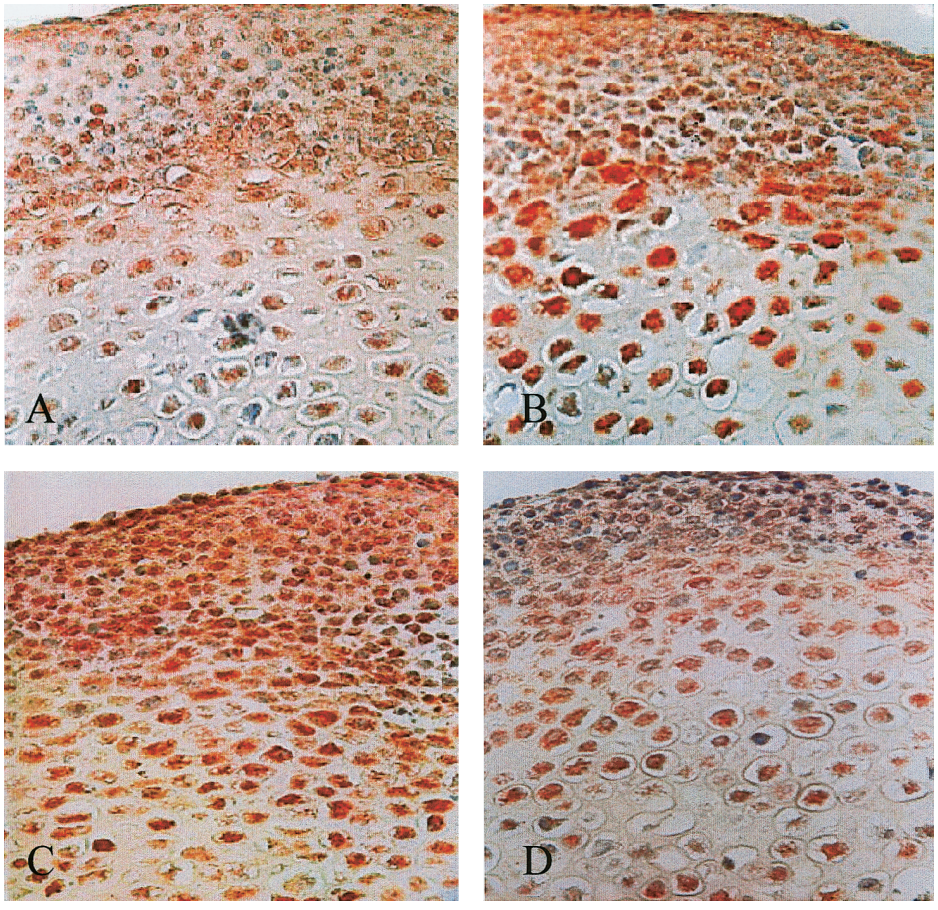

FIG. 9. The effect of leptin on the levels of IGF-I receptors throughout the culture period: time-course study. Condylar cultures incubated under control conditions (A) or with 1.0 μg/ml of leptin for (B) 2, (C) 3, and (D) 4 days were processed routinely for paraffin embedding. Deparaffinized sections were stained with rabbit anti–IGF-I receptor Ab. Maximum stimulatory effect appears after (C) 3 days of treatment.

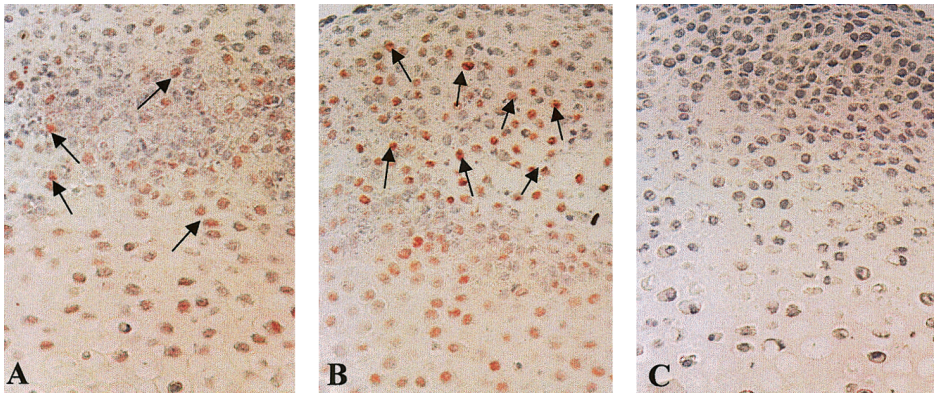

FIG. 10. Effect of IGF-I immunoinhibition on leptin-induced proliferation activity in the mandibular condyle. Condyles cultured for (A) 3 days under control, (B) 1.0 μg/ml of leptin, or (C) 1.0 μg/ml of leptin + anti–IGF-I Ab (1/500) conditions were processed routinely for paraffin embedding. Deparaffinized sections were stained with mouse anti-PCNA antibodies and counterstained with hematoxylin. Positively stained nuclei are red (arrows; magnification $\times$240).

cologic stimulation with insulin, clonidine, arginine, levodopa, glucagon, GH-releasing hormone, and opiate peptides,[31–34] much like children with GH deficiency. Interestingly, evaluations of serum IGF-I levels in obese children have yielded inconsistent findings, with some studies reporting a normal level[35] and others an increase[36] or decrease.[37] The mechanism whereby obese children continue to grow despite the low levels of GH is not known. Several mechanisms have been postulated to explain the "growth without GH" in obesity, such as obesity-induced hyperinsulinemia,[5,38–41] hyperprolactinemia,[5,40,42] induced bioactive but nonimmunoreactive GH molecules,[43] and an increase in free IGF-I levels.[37] The possibility that children with obesity may have an as yet unidentified "circulating factor" that stimulates growth independent of the presence of GH also has been suggested.[44] The results of this study indicate that leptin might have a direct GH-independent effect on the bone growth centers.

Like in adults, serum leptin concentrations in children are directly proportional to the level of adiposity.[12] In humans, obesity is associated with central resistance to

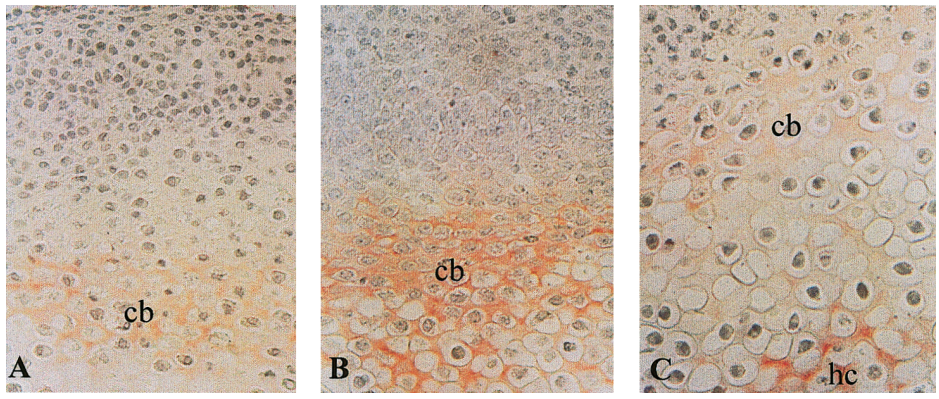

FIG. 11. Effect of IGF-I immunoinhibition on leptin-induced differentiation activity in the mandibular condyle. Condyles cultured for (A) 3 days under control, (B) 1.0 μg/ml of leptin, or (C) 1.0 μg/ml of leptin + anti–IGF-I Ab (1/500) conditions were processed routinely for paraffin embedding. Deparaffinized sections were stained with mouse anti-type II collagen antibodies and counterstained with hematoxylin. (B) Leptin increases the positive staining within the matrix of the chondroblastic zone (cb), and (C) anti–IGF-I blunts this effect leaving only remnants of staining within the hypertrophic zone (hc; magnification $\times$240).

circulating leptin. A differential sensitivity between the center (hypothalamus) and periphery (epiphyseal growth plate) to the effect of leptin might serve as an attractive explanation for the accelerated growth in children with obesity.

In this study, no attempt was made to study the cellular signaling of leptin in the growing chondrocytes and the involvement of the activation of JAK-STSAT system in the process.[16] We found that leptin increased the immunohistochemically positive staining of IGF-I receptor within the chondrocytes and the progenitor cells. This direct in vitro effect of leptin on the activity of the IGF system was independent of changes in GH levels. The increment in the abundance of IGF-I receptor suggests that the IGF system might be involved, at least partially, in mediating the effect of leptin on the growth center of the mandibular condyle. The principal role played by the IGF-I system in mediating the stimulatory effect of leptin on the condylar growth was indicated further by the blocking the IGF-I in the leptin-treated culture. Immunoinhibition of IGF-I completely abolished the effect of leptin on both proliferation, studied by the production of PCNA, and differentiation, studied by the excretion of type II collagen. Further studies are needed to verify the direct interaction between leptin, IGF, and the binding proteins and receptors in the bony growth centers. This study was conducted to investigate the peripheral effect of leptin on the mice mandibular condyle. The applicability of leptin's peripheral activity in mice to human physiology should be studied further, especially in light of the known difference in leptin's central activity between mice and man.

In summary, leptin apparently acts as a skeletal growth factor with a direct peripheral effect on the skeletal growth centers. Some of the effect leptin had on the growing bone may be mediated by the IGF system through an increase in local IGF-I receptor production. We speculate that the high circulating levels of leptin in obese children contribute to their accelerated growth.

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