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Human Articular Chondrocytes Express Functional Leptin Receptors

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The effects of leptin hormone are mediated by interactions with several physiological regulatory systems and the cytokine network, and by targeting cells directly. The leptin receptor is a member of the class I cytokine receptor family, and its signal transduction resembles that induced by many cytokines. We demonstrated that serially cultured human articular chondrocytes possess the leptin receptor (Ob-R), and that this receptor was present on chondrocytes in native human cartilage. In cultured chondrocytes we detected mRNA for the functional isoform of leptin receptor (Ob-Rb or Ob-R_L), and it was revealed that ligand binding resulted in phosphorylation of signal transducers and activators of transcription, namely STAT1 and STAT5. Chondrocytes stimulated with leptin exhibited an increased proliferation and an enhanced synthesis of extracellular matrix (proteoglycans and collagen). These results indicate that leptin affects cartilage generation directly, which is a novel role for leptin in skeletal growth and development.

Key Words: **autologous chondrocyte transplantation (ACT); cartilage defects; human chondrocytes; leptin, leptin-receptor; Ob-Rb; proliferation; proteoglycan; STAT; tissue engineering.**

Leptin hormone is a 16-kDa protein encoded by the obese (*ob/ob*) gene (1). It is primarily produced by white adipose tissue (2), and serum levels of the hormone correlate directly with adipose tissue mass (3). When adipocytes shrink, less leptin is secreted into circulation, and hypothalamic neuronal receptors are less stimulated. This in turn boosts the release of a neurotransmitter (neuropeptide Y) that increases appetite (4), and simultaneously the release of α-melanocyte-stimulating hormone (α-MSH), which blocks the feeling of hunger, is inhibited (5). Thus, leptin has a pivotal physiological role in regulating food intake and energy expenditure (6).

In addition to hypothalamic receptors, leptin targets a variety of other cell types as indicated by expression of functional receptors in peripheral cells, tissues and organs (7). Bone mass is positively correlated with body fat (8, 9), and there is a positive correlation between bone mass and serum levels of leptin in humans (10). Recently, it was shown that primary adult osteoblasts possess functional leptin receptors (11), and it is disputed whether bone remodelling is centrally or locally regulated (12), in the latter case by a direct effect of leptin on osteoblasts (11).

The leptin receptor (Ob-R) is expressed in several alternatively spliced forms with different cytoplasmatic tails: Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, and Ob-Re. Until now, only the long isoform (OB-R_L or Ob-Rb) has been demonstrated to be functional (13). The leptin receptor is a member of the class I cytokine receptor family that mediates effect of more than 20 different cytokines. Its sequence is most similar to the α-chain of leukemia inhibiting factor (LIF) receptor, granulocyte colony stimulating factor (G-CSF) receptor, and to the gp130 signal-transducing component of interleukin (IL)-6 receptor (13). Signaling by these receptors entails activation of receptor associated kinases of the Janus kinase (JAK) family leading to phosphorylation and activation of the nuclear DNA binding activity of signal transducers and activators of transcription (STATs); STAT1, STAT3 and STAT5 (14–16). In animal studies, exogenous leptin administration promotes production of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), and IL-12 (17), whereas exogenous administration of TNF-α or IL-Iβ results in enhanced levels of serum leptin (18, 19). Hence, leptin is considered a cytokine-like hormone.

Leptin deficient mice (*ob/ob*) exhibit hyperglycemia, hyperinsulinemia, hyperphagia, obesity, infertility, decreased brain size and decreased stature. Leptin ad-

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ministration to these mice led to a significant increase in femoral length, total body bone area, bone mineral content and bone density compared to that of vehicle treated controls (11). Longitudinal bone growth involves endochondral ossification where a scaffold of cartilage develops that is later replaced by bone by the invading osteoblasts, i.e., cells that express functional leptin receptor.

In view of these physiological events and recent findings, the present study was undertaken to investigate whether human articular chondrocytes express functional leptin receptor, and hence clarify whether leptin could have a direct action on chondrocytes whereby cartilage generation and skeletal growth could be affected by the hormone. Moreover, it was aimed to reveal biological effects in these chondrocytes as a result of leptin stimulation *in vitro*.

MATERIALS AND METHODS

RT-PCR. Poly A mRNA from chondrocytes was extracted with Qiagen Direct mRNA kit (Merck Eurolab, Oslo, Norway). cDNA was synthesised by using the SuperScript Preamplification System (Life Technologies Ltd., Paisley, UK), and it was treated with 0.1 unit/l *Escherichia coli* RNase H at 37°C for 20 min. PCR was performed in a 50 μl reaction mixture containing cDNA (derived from 0.5 g mRNA), 1 unit of DyNAZym II DNA polymerase, 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM deoxynucleotide triphosphate mixture, and 10 M of each primer. PCR was performed at 94°C for 5 min (first denaturation), and then at 94°C for 30 s (denaturation), 55°C for 30 s (annealing), and 72°C for 1 min (extension), for a total of 35 cycles with a 10 min final extension at 72°C. PCR products were analyzed by agarose gel (2%) electrophoresis and photographed under UV-light.

Nucleotide sequences of PCR primers were designed to detect intracellular and extracellular domains of the functional leptin receptor (20), and these were: Exon 3 (primer1), 5′-CCTTTTCC-CAGGTGTACTTCTCTG-3′ (sense) and (primer 2) 5′-CACCATCC-AGGTTGTCTTTAGGAG-3′ (antisense), 285-bp fragment expected; Exon 20 (primer 3) 5′-GTGGTCCTCTTCTTTTGGAGCC-3′ (sense) and (primer 4), 5′-AGCCCTTGTTCTTCACCAGTTTC-3′ (antisense), 289-bp fragment expected. To test the quality of the mRNA, the presence of APRT-gene transcripts was assayed. For APRT-PCR the following primers were used: 5′-CCCGAGGCTTCCTCTTT-GGC-3′ (sense), and 5′-CTCCCTGCCCTTAAGCGAGG-3′ (antisense), corresponding to the sequences 1940 to 1959 in exon 3, and to the sequences 2725 to 2744 in exon 5 of the adenine phosphoribosyl transferase (APTR) gene, (GenBank Accession No. Y00486), respectively. Contaminating DNA will generate a 800-bp fragment, while mRNA will generate a 300-bp fragment. All reactions were run using a Perkin–Elmer 9600 GeneAmp thermocycler (Perkin–Elmer, Cambridge, UK).

Immunocytochemistry/immunohistochemistry. Identification of the leptin-receptor on serially cultured human chondrocytes was performed with monoclonal mouse anti-human leptin receptor antibody (Cat. No. MAB867, R&D Systems, Minneapolis, MN). Cells were grown on fibronectin coated chamber slides (Cat. No. 177402, Nunc, Roskilde, Denmark) for 24 h. Cultures were then washed twice with PBS (phosphate buffered saline) and fixed for 2 h in cold PBS containing 0.2 M sucrose and 4% paraformaldehyde. After washing twice with PBS, primary antibody diluted 1:250 was added, and incubation was continued overnight at 4°C. After rinsing in PBS, sections were incubated for 4 h at 4°C with secondary antibody (biotin-rabbit anti-mouse IgG, Zymed, San Francisco, CA) until FITC-conjugated streptavidin (Zymed, San Francisco, CA) was added. Negative controls were performed by omitting primary antibody in this procedure.

The labeled cell cultures were examined in a Zeiss Axiophot photomicroscope equipped with phase contrast and incident-light fluorescence optics (Carl Zeiss, Oberkochen, Germany), and micrographs were taken with Kodak Ektachrome EP1600 film.

Sections of a cartilage biopsy were arranged to study whether leptin receptor was present *in situ*. A piece of surplus cartilage, taken from a low-weight bearing area of a knee-joint subjected to chondrocyte transplantation, was used to develop slices of cartilage to be studied. Paraformaldehyde (4%) containing 0.2 mol/L sucrose in PBS was used as fixative, and after 48 h the biopsy was embedded in paraffin and sectioned at 5 μm thickness onto poly-L-lysine (0.01%, Sigma-Aldrich Sweden AB, Stockholm, Sweden)-coated slides. Sections were deparaffinized by xylene and graded alcohol washes, and immersed in distilled water. Thereafter sections were incubated in PBS containing 1% bovine serum albumin for 30 min following incubation with monoclonal antibody (diluted 1:100) overnight at 4°C. After rinsing in PBS, sections were incubated for 45 min with secondary biotinylated antibody, and thereafter incubated for 2 h with FITC-conjugated streptavidin. Micrographs were taken as described above.

Acquisition of chondrocytes. Human articular chondrocytes were obtained from patients subjected to autologous chondrocyte transplantation (ACT). The patients, aged 24–45 years, suffered from focal cartilage damage in knee-joints, and they participated with informed consent. After a clinical evaluation, arthroscopy of knee-joints were performed, and when lesions were judged suitable for ACT, biopsies were taken from the articular surface at a low-weight bearing area of the joint (21). Subsequently, the biopsies were enzymatically digested and cells were serially passaged for 3 weeks to obtain a sufficient cell-number for transplantation. Surplus cells were cryopreserved and later used in experiments after further expansion of the cultures. In some cases, cryopreservation could be omitted and surplus cells were continuously cultured for pending experiments.

Culture medium. The medium used was DME/F12 (Cat. No. 3-821-35, Imperial Laboratories Ltd., Andover, UK), supplemented with 62 μg/ml ascorbic acid (Cat. No. A-4034, Sigma, St.Louis, MO), and 50 μg/ml gentamycin (Cat. No. G-1264, Sigma). Powdered basal medium (5.7 gr) was added 500 ml sterile water (Cat. No. 883314, Pharmacia, Stockholm, Sweden), and 2.2 g/l $NaHCO_3$ (Cat. No. 1.06329.1000, Merck). Osmolality was adjusted to 290 mOsm/l by using a cryoscopic osmometer (CryoStat, Gonotec GmbH, Berlin, Germany). Subsequently, 280 μl H_2SO_4 (Merck, Darmstadt, Germany) was added. After sterile filtration (0.2 μm) 10% sterile filtered (0.2 μm) autologous serum was added.

Cell cultures. Cells were stored by freezing in an automated cell freezer (Kryo 10, Planer, London. UK) and kept at −180°C (Taylor–Wharton, Cryostorage System, Camp Hill, PA) until experiments (<12 months). The freezing medium was similar to that used during cultivation, except for 5% of the additive Cryoserv (Baxter Research Medical, Irvine, CA). The experimental set-up initially involved thawing of cells and removal of cryopreservative by two consecutive washings. The ampoule contents were transferred to sterile tubes with warm medium supplemented with 10% autologous serum, and the pellet was resuspended in fresh medium after centrifugation at 200 g. Thereafter, cells were resuspended in 5 ml fresh medium after a second wash, and subsequently transferred to 250 ml culture vessels (Cat. No. 3108, Falcon, BD Bioscience, Stockholm, Sweden) kept in humidified air containing 5% CO_2. Cultures were further expanded by trypsinisation (Cat. No. T-3924, Sigma), and after repeated washing, transferred to 500 ml culture vessels (Cat. No. 3110, Falcon). Experiments were performed when an appropriate cell number was achieved as judged by microscopy.

Assessment of cell proliferation. Incorporation of tritium labeled thymidine (methyl-^{3}H-thymidine, Cat. No. NET-027Z, NEN Life Science Products, Boston, MA) was used as marker of cell proliferation.

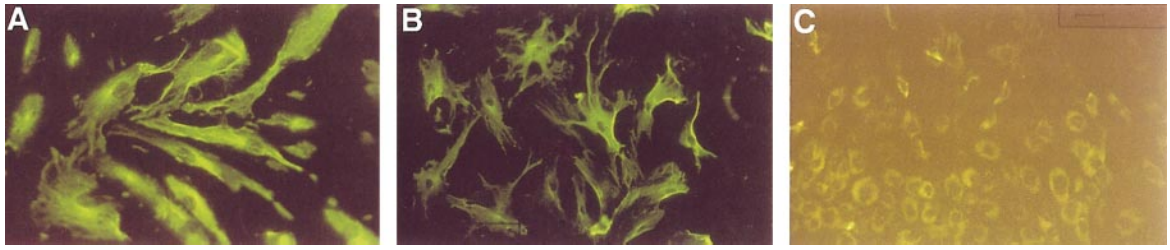

FIG. 1. Micrographs of cultured human articular chondrocytes stained with monoclonal mouse anti-human leptin receptor antibody (A and B), and without primary antibody (C). Magnification is 40×. Micrographs A and B show positively stained chondrocytes.

Cell cultures were trypsinised, washed twice, and then cells were seeded in 96-well microtiter plates at a density of 1.0×10^4/well. The medium was supplemented with 2 μCi/ml thymidine, added at a total volume of 100 μl/well. After 4 days of challenge with leptin, radioactivity was determined by using direct β-counting ($Micromate_{196}$/$Matrix_{96}$, Packard Instrument Company, Meriden, CT); material in microwells was captured onto a filter (Packard) using a harvester ($Micromate_{196}$), and counts per minutes (cpm) were recorded by a computer after reading in the counter ($Matrix_{96}$).

Assessment of proteoglycan synthesis. Incorporation of ^{35}S-sulphate (Cat. No. NEX041H, NEN, Life Science Products) was used as marker of proteoglycan synthesis. Cells were collected by trypsinisation, thereafter they were washed twice and seeded in 96-well microtiter plates (Cat. No. 3412, Falcon) at a density of 2.5×10^4/well. Medium containing 2 μCi/ml of radioisotope was added at a total volume of 100 μl/well. Challenge with leptin was allowed for 4 days until material in wells was captured onto filters as above, and radioactivity was read by direct β-counting as above.

Assessment of collagen synthesis. Incorporation of tritium labeled proline (Cat. No. NET-323, NEN, Life Science Products) was used as marker of collagen synthesis. Experimental set-up and measurement of radioactivity was identical to that used to assess proteoglycan synthesis.

Western blotting of phospho-STATs. Intracellular signal transduction triggered by leptin was investigated by immunoblotting of phosphorylated STATs (Signal Transducers and Activators of Transcription) by using Phospho-Stat Antibody Sampler (Cat. No. 9914, Cell Signaling Technology, Beverly, MA). Antibodies raised in rabbits toward Phospho-STAT1, Phospho-STAT3, Phospho-STAT5, and Phospho-STAT6 were used to detect leptin receptor-mediated phosphorylation of STATs.

Cell cultures were arranged in two groups, one challenged with leptin and a negative control with no leptin. Cells were grown in 10% autologous serum in 50 ml culture vessels to a density of 1 million cells per vessel. Thereafter, the cells were washed twice in phosphate buffered saline (PBS), and supplemented with 0,10% autologous serum. After two days, cultures were challenged with 600 ng/ml recombinant human leptin (cat. no. 398-LP, R & D Systems, Minneapolis, MN, USA) for 30 min and then washed twice in PBS. The cells were harvested directly in 200 μl SDS-sample buffer (62,5 mM Tris–HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0,1% w/v bromphenol blue). Subsequently, the solution was heated to 100°C for 5 min. Twenty-five microliters of each extract was separated by electrophoresis in NuPage Mes SDS running buffer (Cat. No. NP 0002, Invitrogen, Groningen, The Netherlands) at 200V (constant) using 110–125 mA per gel (NuPage 4–12% BIS-Tris Gel, Cat. No. NP 0322, Invitrogen) for 40 min. Thereafter, electroblotting was performed by electrotransfer onto PVDF-membranes (Immobilin-P, Millipore, Bedford, MA) and by incubation in blotting buffer (5.8 g Tris base, 29 g glycin, 800 ml water, 200 ml methanol). The blots were blocked with 5% nonfat dry milk/0.1% Tween 20 for 1 h at room temperature, and then incubated with primary antibody overnight at 4°C in 5% BSA/0.1% Tween 20. Phospho-STAT antibodies diluted 1:1000 were used, and the untreated cultures that served as negative controls. A prestained protein marker (Cat. No. 7707S, New England Biolabs, Frankfurt am Main, Germany) was used to control the efficacy of electophoresis, and a biotinylated protein marker detection pack (Cat. No. 7726S, Cell Signaling Technology) was used to assess molecular weight (kDa) of proteins. Antibodies toward various non-phosphorylated STATs (Cat. No. 17-176, Upstate Biotechnology, Waltham, MA) were used to detect the constitutive expression of STAT-proteins (STAT1, 2, 3 and 5).

After washing (3×) in blocking buffer, the membranes were incubated with HRP (horseradish peroxidase)-conjugated anti-rabbit IgG antibodies (from the sampler-kit, diluted in blocking buffer 1:30,000) for 1 h at room temperature. Anti-biotin antibodies (HRP-conjugated) were used to detect the biotinylated protein marker, after which substrate (CDP-Star) was added and the blots developed.

RESULTS

Immunocytochemistry/Immunohistochemistry

Micrographs of cultivated chondrocytes showed that cells were stained well in presence of primary antibody compared to that of controls (Fig. 1). This indicates that the leptin receptor was present on serially cultured human chondrocytes.

Similarly, sections of cartilage tissue showed brightly stained cells in presence of primary antibody compared to the unstained controls, indicating that human chondrocytes express leptin receptor *in situ* (Fig. 2).

RT-PCR

Cells from four individuals were separately analysed for transcripts for the functional form, i.e., the long form of leptin receptor (Ob-R_L or Ob-Rb) by RT-PCR analysis. The cDNAs detected by gel electrophoresis indicate that mRNAs corresponding to 285 bp (Exon 3) and 289 bp (Exon 20) were present (Fig. 3). These primers were designed to detect exons coding for the extracellular and intracellular domain of the long form of leptin receptor, respectively. Lanes reflecting runs without the respective primers, i.e., negative control, were negative. These results demonstrate that cultured chondrocytes express functional leptin receptor.

Western Blotting for Phospho-STATs

In Western blot studies of cultured chondrocytes challenged for 30 min with 600 ng/ml leptin, bands occurred

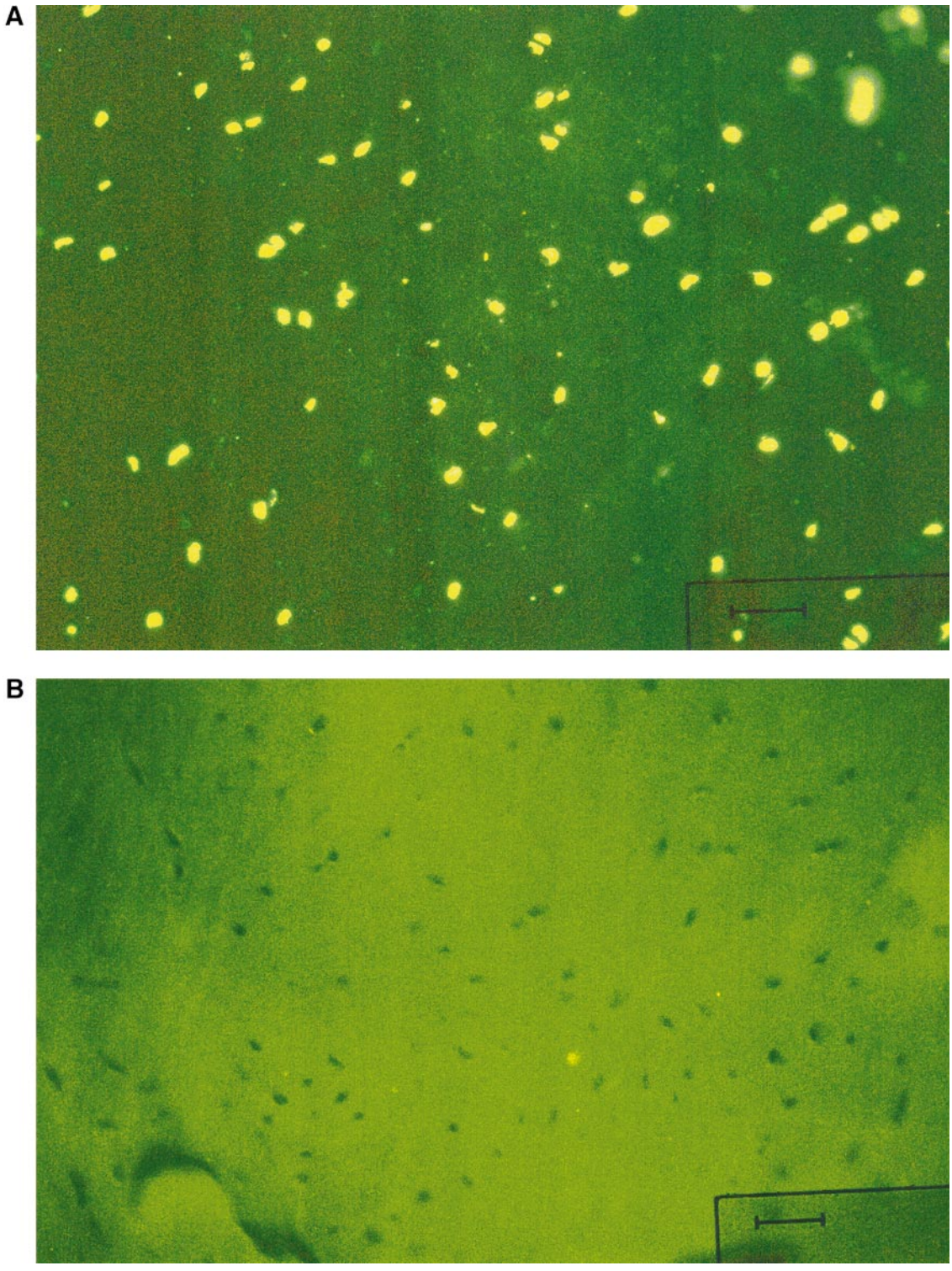

FIG. 2. Section of human articular cartilage tissue stained with monoclonal mouse anti-human leptin receptor antibody (A). Negative control (B) had no primary antibody. The chondrocytes are localised in lacunae within the cartilage, and they are brightly stained in section A. The scalebars are 80 μm.

in lanes where anti-Phospho-STAT1 antibodies were used (Fig. 4A, lanes 1, 2 and 3) corresponding to a protein in the range 76–105 kDa. This suggests a protein identical to Phospho-STAT1 that should migrate to the range 80–90 kDa. The negative control lanes, i.e., cultures that had no leptin (Fig. 4A, lane 5 and 6), were unstained.

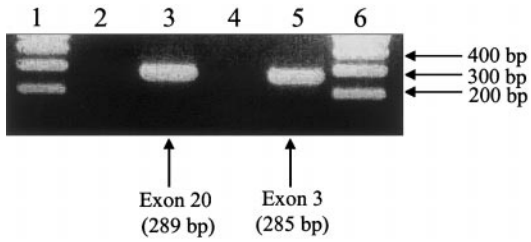

FIG. 3. RT-PCR analysis for the expression of the functional leptin receptor (Ob-Rb) in cultured human articular chondrocytes. PCR products occurred in lanes 3 and 5 which reflect runs with primers used to detect exon 20 (289 bp) and exon 3 (285), i.e., exons coding for the intracellular and extracellular domain of the long form of leptin receptor, respectively. Lanes 2 and 4 reflect runs without the respective primers (negative control).

Similarly, when using anti-Phospho-STAT5 antibodies, a protein occurred in the range 76–105 kDa (Fig. 4B, lanes 1, 2 and 3), reflecting phosphorylated STAT5 that should migrate to the range 80–90 kDa. Cultures that had no leptin were unstained (Fig. 4B, lane 5 and 6), and blotting of STAT3 and STAT6 did not occur (results not shown). Blotting of nonphosphorylated STATs using the antibody sampler kit (antibodies toward STAT1, 2, 3, and 5) showed that nonphosphorylated STAT1, 3 and 5 were present (results not shown), which indicate a constitutive expression of these STATs in human chondrocytes.

Effect of Leptin on Proliferation, and Synthesis of Proteoglycans and Collagen

The effect of leptin on cell proliferation was investigated by measuring incorporation of radio-labeled thymidine in cells challenged with a series of leptin concentrations. Cell cultures from six individuals were

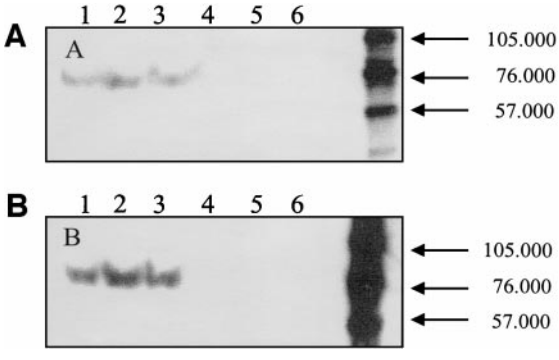

FIG. 4. Western blotting of phosphorylated STAT1 (A) and phosphorylated STAT5 (B). Following starvation for 48 h, human articular chondrocytes were challenged for 30 min with 600 ng/ml recombinant human leptin. Thereafter the cells were harvested and subjected to electroblotting. Rabbit anti-human-phospho-STAT1/STAT3/STAT5/STAT6 were used, and bands occurred where antibodies toward STAT1 and STAT5 were present. It can be seen that bands are present in the lanes 1, 2 and 3, which reflect cell cultures challenged with leptin. Lane 4 reflects an empty lane, whereas lanes 5 and 6 reflect cell culture that had no leptin (negative control).

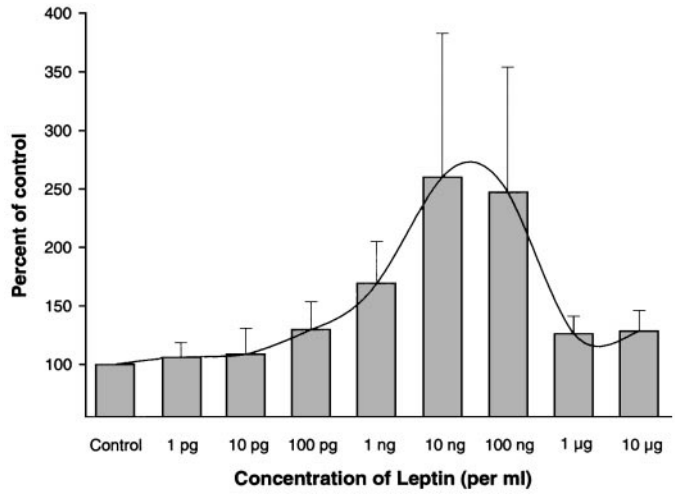

FIG. 5. The effect on cell proliferation of recombinant leptin detected as incorporation of ^{3}H-thymidine. Human articular chondrocytes were seeded at a density of 1.0×10^{4} cells/well in microtiter plates and grown in medium supplemented with 10% autologous serum for 4 days during challenged with a series of leptin concentrations. The results are expressed as percentage of the radioactivity-counts detected in the controls, i.e., cells that had no leptin. The bars are means ± SEM of quadruplicate measurements of six separate experiments, i.e., cells from six different individuals.

investigated separately, and leptin was added in concentrations ranging from 1 pg/ml to 10 μg/ml, controls without leptin were also investigated. Radioactivity was detected after four days of challenge with leptin, and radio-labeling of treated cells was compared to that of untreated cells (controls). Wells containing only medium supplemented with radiolabeled thymidine served as blanks. Proliferation was calculated as percentage radioactivity of that observed in controls in each experiment (patients served as own controls).

It can be seen in Fig. 5 that leptin affected proliferation of chondrocytes in a biphasic manner, and that it was a marked stimulatory effect in the range 10–100 ng/ml. In raw data, counts per minutes (cpm) in controls ranged from approximately 1,000–8,000 cpm, which indicates a marked variation in labeling, which in turn reflects a basal cell-growth that varies between individuals.

The effect of leptin on proteoglycan synthesis was detected by measuring incorporation of ^{35}S-labeled SO_4 in cultures. The experimental set-up was paralleled and identical to that used to measure proliferation, except for the higher cell number used to measure proteoglycan synthesis. Each of the six individuals served as own control by expressing radioactivity in treated cultures as percentage of that in untreated cultures in each experiment. Figure 6 shows that leptin enhanced the incorporation of radioisotope in a biphasic manner with an optimum between 0.1–1.0 ng/ml. A marked variation in labeling of controls was observed

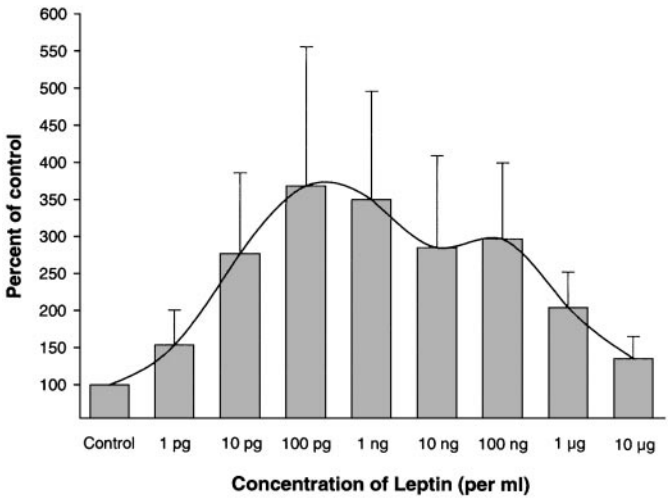

FIG. 6. The effect of recombinant leptin on proteoglycan synthesis in human articular chondrocytes detected as rate of incorporation of radio-labeled sulphate ($^{35}SO_4$). The cells were seeded (2.5×10^4 cells/well) in microtiter plates and challenged for 4 days with the isotope in medium supplemented with 10% autologous serum. The results are expressed as percentage of the radioactivity-counts detected in the controls, i.e., cells that had no leptin. The bars are means ± SEM of quadruplicate measurements of six separate experiments, i.e., cells from six different individuals.

(raw data range 2,600–7,300 cpm) reflecting variance among the individuals.

The effect of leptin on collagen synthesis was detected by measuring incorporation of ^{3}H-proline in cells. An identical experimental set-up and labeling protocol was used as applied to the aforementioned experimental series, except that there were three experiments. It can be seen (Fig. 7) that leptin enhanced collagen synthesis markedly with an optimum in the range 10 pg to 100 pg/ml, and that the effect was biphasic. Here we also observed variance between individuals in basal labeling with the radioisotope.

DISCUSSION

The product of the *ob* gene was positionally cloned in 1994 (1). Discovery of the normal hormone product of the ob locus, leptin, and the cloning of its receptor (Ob-R) followed in short order (22). Initially, leptin was considered a satiety factor that primarily regulated food intake and energy expenditure, as indicated by the appearance of the animals deficient in leptin production, the *ob/ob* mice, and by the receptor deficient animals such as fatty Zucker rat (*fa/fa*) and the *db/db* mice. However, recent research has revealed that leptin has a multitude of other effects mediated by its interaction with several physiological regulatory systems and by targeting cells directly. The leptin receptor is a member of the class I cytokine receptor family and its signal transduction utilises the JAK-STAT pathway, which is activated by several cytokines (23, 24).

A central role in bone remodelling was recognised by the observation that leptin deficient mice (*ob/ob*) and leptin-receptor deficient mice (*db/db*) had a higher bone mass than wild-type mice. Intracerebroventricular injection of leptin in *ob/ob* mice was reported to rescue their phenotype despite undetectable levels of leptin in sera of these animals. The failure to demonstrate expression of functional leptin receptor ($Ob\text{-}R_L$) on differentiated murine osteoblasts led in part to the hypothesis that leptin affects bone formation by involving a central component, most likely the hypothalamus (12, 25). However, functional leptin receptors were recently reported to be present on human primary adult osteoblasts, suggesting a direct effect of leptin on these cells (11). These authors also revealed leptin receptor expression by porcine chondrocytes. Furthermore, it was shown that chondrocytes in skeletal growth centers contained specific binding sites for leptin by using two mouse models for endochondral ossification: the mandibular condyle and the humerus growth plate (26). Together these findings indicate that leptin also may target osteogenic and chondrogenic cells directly.

Bone growth and bone formation are dependent on a concerted action of three mesenchymal cells; chondrocytes, osteoblasts and osteoclasts. Longitudinal bone growth involves endochondral ossification where a scaffold of cartilage develops that is later replaced by bone, as a result of invading osteoblasts. Thus, it can be speculated that osteoblasts and chondrocytes could be subjected to similar regulatory mechanisms regarding production of their respective extracellular matrices.

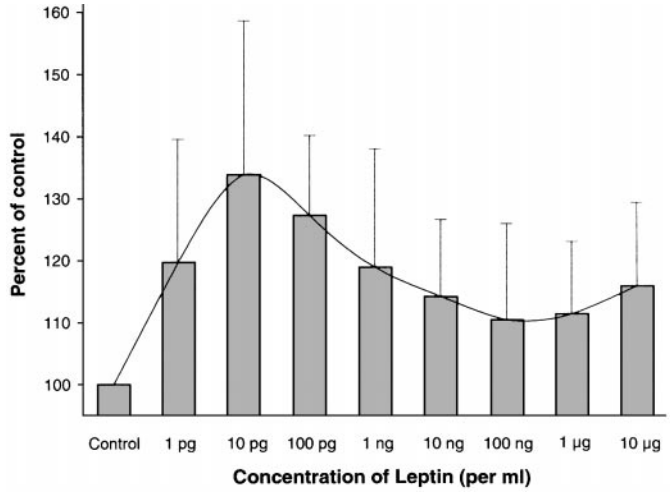

FIG. 7. The effect of recombinant leptin on collagen synthesis. Human articular chondrocytes (2.5×10^4 cells/well) were cultivated in microtiter plates for 4 days in medium supplemented with 10% autologous serum, and the incorporation of ^{3}H-proline was used as a marker of collagen synthesis. The results are expressed as percentage of the radioactivity counts detected in the controls, i.e., cells that had no leptin. The bars are mean ± SEM of quadruplicate measurements of three separate experiments, i.e., cells from four different individuals.

Increased weight as a result of larger fat storage should under a normal physiological condition require stronger skeletal construction to withstand a heavier strain.

Functional leptin receptors have been identified in several organs, tissues and cell types including mesenchymal cells such as platelets, hematopoietic progenitors (CD^{34+}-cells), bone marrow stromal cells, lymphocytes, and adipocytes, in addition to osteoblasts (7, 11). A proliferative effect of leptin has been reported in several studies, such as hematopoiteic multilineage progenitors (27), gastric mucosa cells (28), pancreatic β-cells (29), T-lymphocytes (30), and tracheal epithelial cells (20). In the present study we demonstrated expression of functional leptin receptor on cultured human chondrocytes, suggesting that leptin may have a central role in regulating cartilage synthesis as indicated by its proliferative effect on chondrocytes. This is further supported by the findings that leptin had a stimulatory effect on synthesis of extracellular matrix as determined by collagen and proteoglycan synthesis in cell cultures that were grown to confluence, a condition that inhibits cell proliferation in normal cells.

Functional leptin receptors have signaling capabilities of IL-6R (13). Signaling by this receptor entails, among other things, the activation of receptor-associated kinases of the Janus kinase family that contribute to the phosphorylation and activation of the DNA binding activity of STAT1, STAT3, and STAT5 (14–16). In studies of different cell lines, signal transduction by Ob-Rb was accompanied by phosphorylation of STAT1, STAT3 and STAT5 (13), whereas only Phospho-STAT3 could be detected in the hypothalamus of mice (31). Leptin failed to induce phosphorylation of STAT3 in primary osteoblasts (25). Similarly, in our experiments Phospho-STAT3 and Phospho-STAT6 were not detected with the antibodies we used (results not shown). On the other hand, phosphorylated STAT1 and STAT5 were induced by leptin indicating that human chondrocytes utilise these STATs as signal transducers.

In humans, the circulating level of leptin is normally 1–10 ng (32, 33). Typically in the present experiments, leptin exhibited a bifasic effect with an optimum between 0.1–100 ng/ml. This may be within a physiological level regarding biologic effects in tissues, indicating that the reduced effect observed at the higher concentration of leptin could be a result of a negative feedack-loop, i.e., receptor inhibition. It is well established that cytokine-signaling pathways are negatively regulated, and several such regulators have been identified (34). In mouse hypothalamus expression of SOCS3 (suppressors of cytokine signaling) was induced by leptin injection (35, 36). They demonstrated that the Agouti mouse, which exhibits leptin resistance and obesity, has an elevated level of SOCS3 that may account for the resistance to leptin action. Hence, a decline in proliferation and matrix synthesis at the higher levels of leptin as observed here, can be explained by negative feedback due to factors such as SOCS.

In our study we demonstrated that chondrocytes express functional leptin receptors *in situ* by immunohistochemistry. It can not be claimed that this receptor is the functional type of leptin receptor since the primary antibody detects the extracellular part of the receptor and thereby does not distinguish between the different cytoplasmatic tails that constitute the isoforms. In case this receptor proves to be identical to the long form of the receptor found on serially cultured chondrocytes, we suggest that leptin affects both generation and regeneration of cartilage tissue.

In several rodent models of obesity including db/db, fa/fa, yellow (Ay/a), and those induced by gold thioglucose, monosodium glutamate, and transgenic ablation of brown adipose tissue, leptin mRNA expression and the level of circulating leptin are increased, suggesting resistance to one or more of its actions (37). Leptin resistance in hypothalamic neurons, which indirectly regulates fat deposits and in turn the serum level of leptin, combined with chondrocytes and osteoblasts that respond normally to leptin action, could explain the higher bone mass observed in leptin deficient mice. The leptin deficient mice (*ob/ob*) exhibit hyperglycemia, hyperinsulinemia, hyperphagia, obesity, infertility, decreased brain size and decreased stature. Leptin administration to these mice led to a significant increase in femoral length, total body bone area, bone mineral content and bone density compared to vehicle treated controls (11), indicating that a developmental defect can be readily reversed upon leptin administration.

Taken together, these results show that leptin targets chondrocytes directly and that leptin may stimulate cartilage generation, which suggests a novel role for leptin in human skeletal growth and development. This suggests future potential in treatments for diseased human cartilage such as promotion of cell growth *in vitro*, or cartilage tissue generation both *in vivo* and *in vitro*.

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