Journal of Cellular Biochemistry 94:50–57 (2005)

High Affinity Leptin Receptors Are Present in Human Mesenchymal Stem Cells (MSCs) Derived From Control and Osteoporotic Donors

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Abstract There are disparate observations on central and peripheral effects of leptin, but several studies consistently support its role as a link between fat and bone. Bone marrow stroma contains mesenchymal stem cells (MSCs), which differentiate into osteoblasts and adipocytes, among others. In this study we assessed the expression of leptin receptors protein in MSCs from control and osteoporotic postmenopausal donors and their change during osteogenic and adipogenic differentiation. Also, we assessed the effects of leptin on osteogenic and adipogenic differentiation of these cells. We demonstrated high affinity leptin binding ($K_D = 0.36 \pm 0.02$ nM) in both types of cells. Binding was very low under basal, but increased significantly (2–3 times) through osteogenic and adipogenic differentiation. Osteoporotic MSCs showed lower leptin binding capacity than control cells at an early osteogenic and adipogenic differentiation time, which could restrict cell sensitivity to the protective action of leptin. In this regard, we observed that leptin significantly inhibited adipocyte differentiation in control but not in osteoporotic MSCs, while it exerted a low stimulatory effect on calcium deposition (10%–20%) in both types of MSCs cells. In summary, we report the presence of high affinity leptin receptors on control and osteoporotic MSCs, which were modified distinctly by osteogenic and adipogenic stimulation and a direct and distinct effect of leptin on both type of cells. J. Cell. Biochem. 94: 50–57, 2005. © 2004 Wiley-Liss, Inc.

Key words: leptin receptor; leptin; mesenchymal stem cell; osteogenic differentiation; adipogenic differentiation; osteoporosis

Several clinical studies have demonstrated that body fat and bone mass are directly related and that serum leptin levels are increased in obesity and correlate positively with fat mass [Glauber et al., 1995; Khosla et al., 1996]. However, the basis for a link between obesity and the skeletal system is often unclear; leptin has recently emerged as a potential candidate responsible for the protective effects of fat on bone tissue [Considine et al., 1996; Thomas et al., 2000]. Although published data on leptin effects on bone metabolism are apparently contradictory [Thomas and Burguera, 2002], its effects are thought to be largely mediated via the hypothalamus [Karsenty, 2001]. The observation that the leptin receptor is widely expressed throughout the body suggests that leptin may operate directly in peripheral tissues [Tartaglia, 1997; Reseland et al., 2001; Holloway et al., 2002].

The bone marrow stroma contains mesenchymal stem cells (MSCs) that differentiate along the osteogenic, chondrogenic, adipogenic, and marrow stromal lineages [Caplan, 1991; Bruder et al., 1997; Pittenger et al., 1999]. Several in vitro studies indicate that stromal cells are responsive to leptin, which increases proliferation, differentiation to osteoblastic lineage, and the number of mineralized nodules [Takahashi et al., 1997; Thomas et al., 1999; Reseland et al., 2001], but inhibits cell differentiation to adipocytes [Thomas et al., 1999]. These observations suggest that leptin may participate in the regulation of bone mass, but the mechanism by which this occurs remains unclear. Recently, the expression of a functional leptin receptor has been demonstrated in precursor cells of

Grant sponsor: FONDECYT; Grant number: 1020728.

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Received 14 July 2004; Accepted 7 September 2004
DOI 10.1002/jcb.20330

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the osteoblast-lineage [Thomas et al., 1999; Kim et al., 2003] showing that they can be direct targets for leptin. Moreover, it is known that the leptin-induced activation of the MAPK cascade stimulates osteoblastic differentiation [Lai et al., 2001] as well as phosphorylation of peroxisome proliferator-activated receptors (PPARγ). This last event has been shown to inhibit adipogenesis [Hu et al., 1996].

There is evidence suggesting that changes in the functional characteristics of MSCs, or changes in the regulation of the differentiation pathway may have important implications in some osteogenic disorders like human postmenopausal osteoporosis [Bergman et al., 1996; Gimble et al., 1996; Nuttall et al., 1998; Bianco and Robey, 1999; Rodríguez et al., 1999; Rodríguez et al., 2000]. Increased adipogenesis in bone marrow as bone volume decreases in postmenopausal women seems to be an important factor in the fragility of adult bone [Nuttall et al., 1998]. We have previously observed in vitro that MSCs obtained from control and osteoporotic women show differences in their capacity to differentiate into the osteogenic and adipogenic pathways [Rodríguez et al., 1999; Rodríguez et al., 2000]. This phenomenon, coupled with the possibility that osteoblastic and adipocyte differentiation pathways are regulated jointly, points to the importance of marrow adipogenesis in osteogenic disorders [Nuttall et al., 1998; Rodríguez et al., 2000]. Leptin could be one of several factors that modulate the reciprocal differentiation of stromal cells between osteoblastic and adipocytic pathways.

Since, leptin protein receptors have been previously detected only in immortalized human MSC cell lines, the aim of the present study was to characterize leptin binding capacity of primary MSCs derived from control and osteoporotic women and to analyze whether osteogenic and adipogenic cell differentiation affect leptin receptor level. In addition, we studied the direct effect of leptin on osteogenic and adipogenic differentiation of both types of MSCs.

MATERIALS AND METHODS

Subjects

Postmenopausal women aged 65–86 years old, who required bone surgery because of bone fracture at the Trauma Section of Hospital Sótero del Río, Santiago, Chile, were asked to volunteer as bone marrow donors. Written informed consent was obtained from all subjects. Bone marrow was obtained by iliac crest aspiration during surgical procedures [Rodríguez et al., 1999]; ethical approval was obtained from the Hospital Sótero del Río and INTA ethics committees. Bone mineral density (BMD) was measured for each subject within 4 weeks after surgery using dual-energy X-ray absorptiometry (DXA) (LUNAR, Prodigy, General Electric Medical Systems, Madison, WI). Donors were classified as control (six) or osteoporotic (six) according to their BMD value; control donors had BMD higher than −2.5 standard deviations (SD) of the mean BMD for young adults and osteoporotic donors had BMD lower than −2.5 SD, and hip fracture [Raisz, 1997]. Control and osteoporotic donors considered themselves healthy, except for fracture, and were not under glucocorticoid or estrogen replacement therapy. Characteristics of donors are shown in Table I.

Reagents

Tissue culture media and reagents were purchased from either Sigma Chemical Co. (St. Louis, MO) or Gibco/BRL (Gaithersburg, MD); recombinant human leptin (rh-leptin) was purchased by Chemicon International, Inc. (Temecula, CA), and ^{125}I-leptin (2,200 Ci/mmol) by NEN Life Science Products (Boston, MA). Tissue culture plasticware was purchased from Nunc (Naperville, IL).

TABLE I. Characteristics of Donors of Mesenchymal Stem Cells

	Control		Osteoporotic		
Characteristic	Mean ± SD	Range	Mean ± SD	Range	*P*-value
N	6		6		
Age (years)	71.4 ± 3.83	65 to 77	72.3 ± 8.41	62 to 86	0.818
LS T score[a]	−1.22 ± 0.95	0.3 to −2.1	−3.83 ± 1.37	−2.6 to −6.2	0.002
BMD (g/cm^2)[b]	1.057 ± 0.116	0.949 to 1.241	0.742 ± 0.164	0.458 to 0.891	0.002

[a]LS, lumbar spine; T score, number of standard deviations by which an individual value differs from a young adult population.
[b]BMD, bone mineral density.

Cell Preparation and Culture Methods

MSCs were classified as control or osteoporotic according to whether they were derived from control or osteoporotic donors. MSCs were isolated from bone marrow as previously described [Jaiswal et al., 1997]. Briefly, 10 ml of bone marrow aspirate was added to 20 ml of Dulbecco's Modified Eagle medium high glucose (D-MEM) containing 10% fetal bovine serum (FBS) and 10% stock penicillin (10,000 U/ml)–streptomycin (10,000 U/ml) (basal medium), it was then centrifuged to pellet the cells, discarding the fat layer. Cells were suspended in basal medium and fractionated on a 70% Percoll density gradient. The MSCs-enriched low-density fraction was collected, rinsed with culture medium, and plated at a density of 1–2×10^7 nucleated cells/100 mm dish. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. After 4 days in culture, non-adherent cells were removed and fresh culture medium was added. Culture medium was replaced by fresh medium twice weekly. When culture dishes became near confluent, cells were detached by a mild treatment with trypsin (0.25%, 5 min, 37°C) and replated at 1/3, the original density to allow for continued passaging. The experiments described here were performed after the fourth cell passage.

Leptin Binding Assays

MSCs (triplicate 9–12×10^4 cells/35 mm dish), treated or untreated for osteogenic or adipogenic differentiation in the absence of leptin, were washed twice with ice-cold PBS. Binding buffer containing 25 mM HEPES, pH 7.4, 1% (w/v) BSA, and 50 pM ^{125}I-leptin in DME medium, either in the presence or absence of unlabeled rh-leptin was added, and cells were incubated for 4 h at 4°C [Barr et al., 1999]. The medium was removed and cells were washed twice with cold PBS containing 1% (w/v) BSA, and then solubilized with 0.4N NaOH. Bound ^{125}I-leptin was counted in a gamma-counter (Packard, Downers Grove, IL).

Osteogenic Differentiation

MSCs obtained from control and osteoporotic donors (2.5×10^4 cells/well, 24 wells dish) were maintained in osteogenic culture medium (basal medium supplemented with 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid added daily (OS)) for up to 14 days [Rodríguez et al., 1999] in the presence of freshly prepared leptin (1 μg/ml) or vehicle (PBS). The medium was changed twice a week. The ability of MSCs to differentiate into the osteoblastic lineage was evaluated measuring calcium phosphate deposition on the cell layer as a late osteogenic differentiation marker [Hu et al., 2003]. After 10 days in culture, the calcium phosphate crystals deposited on the cell layer were solubilized in 0.5N HCl, and the amount of calcium was measured by atomic absorption spectroscopy (423 nm) [Rodríguez et al., 2002] (Atomic Absorption Spectrophotometer Model 2280, Perkin-Elmer, Norwalk, CT). Results were expressed as μg/well of calcium deposited in the presence and the absence of leptin.

Adipogenic Differentiation

MSCs obtained from control and osteoporotic donors (1×10^5 cells/dish, 35 mm) were cultured in basal and AD. The AD consisted of basal medium supplemented with 1 μM dexamethasone, 10 μg/ml insulin, 0.45 mM isobutyl-methyl-xanthine, and 0.1 mM indomethacin in the presence of freshly prepared leptin (1 μg/ml) or its vehicle (PBS). This was replaced by fresh medium every 4 days. After 14 days in culture, cells were released from the culture dish with a mild trypsin treatment (0.25%, 5 min at 37°C) and counted in a hemocytometer. Cell concentration was adjusted to 1×10^6 cells/ml and MSCs were tested for their adipogenic potential by flow cytometric quantitation of lipid accumulation after exposure to the adipogenic conditions in culture. Cells were placed in freshly diluted Nile Red (1 mg/ml) and analyzed by flow cytometry [Dennis et al., 1999] (FACS-Calibur, Becton Dickinson, Franklin, NJ).

Statistical Analysis

All values are expressed as mean ± SD. Two-sample Student's *t*-test was used to evaluate differences between samples and the respective controls; $P < 0.05$ was considered significant.

RESULTS

Measurement of Leptin Binding Capacity of MSCs

Considering that endogenous leptin membrane receptors in MSCs had not been previously quantified, we studied the leptin binding capacity of both control and osteoporotic MSCs

treated or untreated for osteogenic and adipogenic differentiation. Preliminary studies showed that osteogenic stimulation of cells importantly increased their leptin binding capacity. Therefore, further characterization of leptin binding was done in cells under OS conditions for 3 days. Specific binding of ^{125}I-leptin by cells was measured by competition studies using increasing amounts of rh-leptin, as indicated. The unlabeled leptin dose dependently inhibited the binding to cell monolayers in a monophasic manner ($IC_{50} = 0.4 \pm 0.09$ nM). As shown in Figure 1, a high signal to noise ratio was observed in this total cell binding assay with specific (i.e., 10 nM unlabeled leptin displaceable) ^{125}I-leptin binding representing more than 90% of the total ^{125}I-leptin binding.

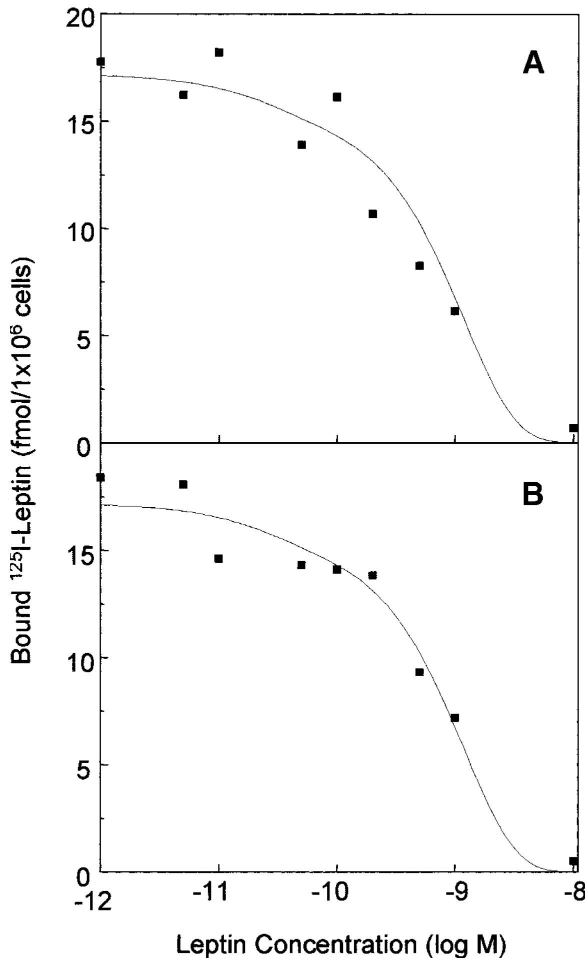

Fig. 1. Representative experiment of competition of ^{125}I-leptin binding to the cell leptin receptor by recombinant human leptin. Triplicate samples of control (**A**) or osteoporotic (**B**) mesenchymal stem cells were incubated with 50 pM ^{125}I-leptin and the indicated concentrations of unlabeled leptin; binding assay was performed as described in Materials and Methods.

Leptin binding exhibited comparably high affinities in both control and osteoporotic MSCs ($K_D = 0.36 \pm 0.02$ nM) and a single binding site by Scatchard analysis of data (not shown).

Figure 2 shows ^{125}I-leptin binding capacity of control and osteoporotic MSCs under both osteogenic and adipogenic differentiation culture conditions. Under basal culture conditions, ^{125}I-leptin binding capacity of MSCs was low and equal in both control and osteoporotic cells at all time tested (data not shown). Under OS conditions, ^{125}I-leptin binding capacity increased significantly and varied over the differentiation

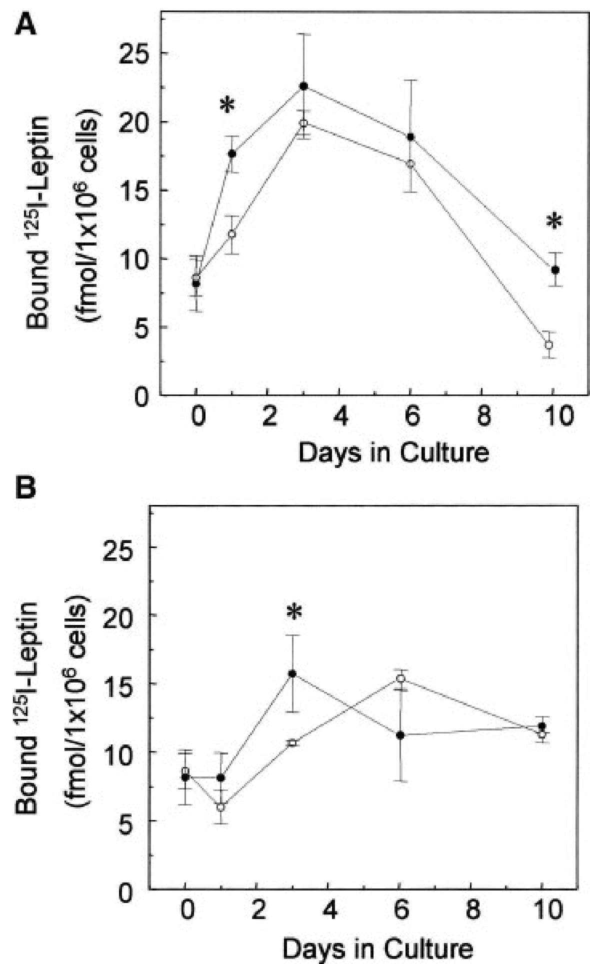

Fig. 2. Leptin binding capacity of mesenchymal stem cells (MSCs) during osteogenic and adipogenic differentiation. Control (filled circles) and osteoporotic (open circles) MSCs were cultured in OS (**A**) and AD (**B**) conditions as described in Materials and Methods. At indicated times leptin binding capacity was measured incubating cells with 50 pM ^{125}I-leptin in the presence or absence of 10 nM unlabeled leptin. Experiments were performed in triplicate from six different samples (three control and three osteoporotics). Results are the mean ± SD. $^{*}P < 0.05$, compared with corresponding osteoporotic value.

period, as appreciated in Figure 2A. In both types of cells, maximum binding capacity was observed after 3 days in osteogenic culture conditions. Afterwards, ^{125}I-leptin binding capacity decreased so that after 10 days of osteogenic differentiation the binding value matched the basal level. A similar pattern was observed for osteoporotic MSCs, but leptin binding capacity was lower in osteoporotic than in control cells during all the differentiation period analyzed. Thus, at day 3 of differentiation ^{125}I-leptin binding capacity for control and osteoporotic cells had increased 2.7- and 2.3-fold, respectively, compared with basal binding.

Adipogenic culture conditions modified also the leptin binding capacity of control and osteoporotic MSCs, but the hormone binding profile was different to that observed through OS differentiation (Fig. 2B). Thus, although the leptin binding capacity of control cells was maximal after 3 days of adipogenic treatment, the values were lower than under osteogenic differentiation conditions. On the other hand, although at the end of the adipogenic differentiation period leptin binding capacity had decreased, it was significantly higher than the basal values. Moreover, osteoporotic cells showed a delayed increase of leptin binding capacity; as a result the maximal value was observed after 6 days of differentiation treatment (Fig. 2B).

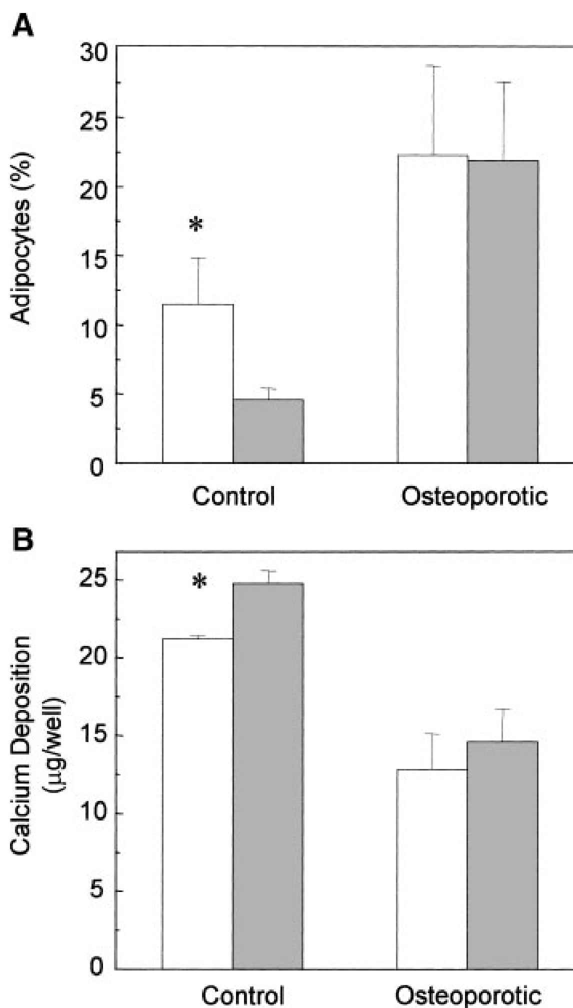

Fig. 3. Effect of leptin on adipogenic and osteogenic differentiation of MSCs. Control and osteoporotic MSCs were cultured in adipogenic or osteogenic conditions, supplemented or not with 1 µg/ml of leptin, as described in Materials and Methods. **A**: Results are expressed as the percentage of adipocytes produced in the absence (white bars) or in the presence (gray bars) of leptin. **B**: Results are expressed as the amount of calcium deposited (µg/well) in the absence (white bars) or in the presence (gray bars) of leptin. Experiments were performed in triplicate in six different samples, three control and three osteoporotic. Results are expressed as mean ± SD. $^{*}P < 0.05$, compared with leptin treated condition.

Direct Effect of Leptin on Adipogenic and Osteogenic Differentiation of MSCs

The ability of control and osteoporotic MSCs to differentiate into adipogenic and osteogenic lineages was studied in the presence or absence of leptin. In the absence of leptin, osteoporotic MSCs exhibited a higher ability to differentiate to adipocytes than the control MSCs (two-fold). On the other hand, the presence of leptin during adipogenic differentiation developed distinctive responses in control and osteoporotic MSCs. Thus, adipogenic differentiation of control MSCs was 40% in the presence of 1 µg/ml of leptin compared with differentiation in the absence of the hormone. However, adipogenic differentiation of osteoporotic MSCs was not affected by the presence of the hormone (Fig. 3A). Therefore, in the presence of leptin, differentiation to adipocytes is five-fold higher in osteoporotic than in control MSCs.

We also observed that the presence of leptin during osteogenic differentiation increased calcium deposition in both control and osteoporotic MSCs by only 10%–20% compared with MSCs cultured in OS conditions in the absence of leptin. The effect was statistically significant only in control MSCs (Fig. 3B). As shown, osteoporotic MSCs deposited less calcium than control MSCs.

DISCUSSION

While the hypothalamus is an important site of leptin action, leptin originates outside the central nervous system, in the adipocyte.

Studies on human bone marrow stromal cell lines have shown that leptin enhances osteoblastic, and inhibits adipocytic differentiation [Thomas et al., 1999]. These cells also expressed both the short and long forms of the leptin receptors (OB-R) [Thomas et al., 1999]. Moreover, cultures of both human bone marrow adipocytes and primary osteoblasts produced relatively large amounts of leptin [Laharrague et al., 1998; Reseland et al., 2001; Gordeladze et al., 2002]. All these observations suggest that leptin may in fact be an important paracrine signaling molecule between adipocytes and preosteoblastic cells in the bone marrow microenvironment. Since the presence of a functional leptin receptor in primary human MSCs had not been measured before, in this study we characterized the binding capacity of control and osteoporotic cells in basal and differentiation conditions.

As a result of differential mRNA splicing, there are several isoforms of the leptin receptor with different lengths and C-terminal sequences. It appears that a short and long membrane-bound receptor and a putative soluble leptin receptor isoforms are actually expressed in various cell types [Chen et al., 1996; Ghilardi et al., 1996; Lee et al., 1996; Kielar et al., 1998; Barr et al., 1999; Aprath-Husmann et al., 2001]. The short and long types of OB-R have been found in bone cells and their precursors [Glauber et al., 1995; Thomas et al., 1999; Reseland et al., 2001; Cornish et al., 2002; Kim et al., 2003]. Variations of OB-R content have been observed depending on cell origin and differentiation stage [Siegrist-Kaiser et al., 1997; Thomas et al., 1999; Glasow et al., 2001], but the OB-R protein content has not been quantitated. The ^{125}I-leptin binding assay in cell monolayers cannot discern among the short and long membrane isoforms of the leptin receptors, but it allows the assessment of the cell membrane binding capacity. In this study the steady-state ^{125}I-leptin binding assay allowed us to characterize high affinity leptin binding in MSCs, with no differences in apparent leptin binding affinities between control and osteoporotic cells. Affinity constant values agree well with those reported for the leptin receptors expressed in other cell types [Liu et al., 1997; Barr et al., 1999]. We observed that under basal conditions both control and osteoporotic MSCs had very low leptin binding capacity during the incubation period studied, but that leptin binding increased significantly under osteogenic or adipogenic conditions. These observations agree with those reported in relation with the expression of OB-R mRNA in conditionally immortalized hMS cell lines [Thomas et al., 1999]; this led the authors to propose that alterations in OB-R expression appear to be induced by reduced proliferation and increased differentiation, rather than by the particular differentiation pathway. However, after measuring ^{125}I-leptin binding protein level, we observed that leptin binding capacity varied according to the specific osteogenic or adipogenic differentiation stimulus.

This study is the first to analyze the leptin binding protein of MSCs and results allow us to conclude that: (1) osteogenic and adipogenic differentiation stimuli significantly increased the leptin binding capacity in MSCs, the response becoming evident very early (in 24 h); (2) the increased leptin binding capacity of MSCs extends throughout the differentiation period studied, revealing a distinct osteogenic or adipogenic patterns for leptin binding capacity; (3) the leptin binding capacity of MSCs is higher during osteogenic than during adipogenic differentiation and; (4) on day 3 of osteogenic or adipogenic differentiation, at which the highest leptin binding values were measured, osteoporotic MSCs showed lower leptin binding capacity than control MSCs.

Little is known about the regulation of any leptin receptor protein isoform. The long form of OB-R has been detected in both early and lineage-restricted hematopoietic progenitors, in the placenta and in different fetal tissues, as well as in mature white and brown adipose tissues [Bennett et al., 1996; Hoggard et al., 1997; Siegrist-Kaiser et al., 1997]. Fasting has been only recently reported to be associated with increased expression of mRNA for the long form of OB-R in the hypothalamus [Baskin et al., 1998], while leptin administration had a modest inhibiting effect on OB-R mRNA in immortalized hMS under adipogenic conditions [Thomas et al., 1999]. Considering that in this study increased leptin binding was observed shortly after triggering differentiation of MSCs (24 h), it seems appealing to consider that factor(s) included in the differentiation media, such as glucocorticoids, could be direct regulators of leptin membrane receptor(s).

We had previously observed that MSCs derived from control and osteoporotic donors show

differences in proliferation rate, IGF-1 responsive capacity, collagen synthesis capacity, and their ability to differentiate towards the osteogenic and adipogenic lineages, [Rodríguez et al., 1999; Rodríguez et al., 2000]. In the current study, we also analyzed whether leptin had a direct effect on MSCs obtained from control and osteoporotic women during osteoblastic and adipocytic differentiation.

Results showed that control and osteoporotic MSCs displayed a distinct response to leptin during their osteogenic and adipogenic differentiation. Thus, leptin significantly inhibited adipocyte differentiation in control cells (60%), but it did not affect adipogenic differentiation of osteoporotic cells. On the other hand, this hormone exerted a similar and low stimulatory osteogenic effect on both control and osteoporotic cells. This effect of leptin on control MSCs agrees with data obtained in immortalized human marrow stromal cell lines [Thomas et al., 1999]. The suggested protective effect of leptin against osteoporosis, could be expressed at the cell level by inhibition of adipocyte differentiation, favoring differentiation towards the osteogenic lineage [Thomas et al., 1999; Thomas and Burguera, 2002]. Since, in osteoporotic MSCs the adipogenic differentiation seems to be stimulated [Rodríguez et al., 2000], and leptin inhibitory action on that differentiation pathway seems blunted, it could be suggested that the protective effect of leptin vis a vis osteoporosis could be restricted to healthy control or early osteopenic cells.

In summary, we observed that the binding capacity of OB-R increased after triggering osteogenic or adipogenic cell differentiation, and that binding capacity of OB-R varied showing a defined pattern (osteogenic or adipogenic) throughout the period studied. We found that during osteogenic and adipogenic differentiation, osteoporotic MSCs had lower leptin binding capacity than control cells, showing less sensitive to leptin action at an early differentiation time. In addition we report a direct leptin effect with different results in control and osteoporotic human MSCs. Thus, in control cells leptin exerted both osteogenic and antiadipogenic effects, but in osteoporotic cells the antiadipogenic effect of leptin disappeared.

Taken together, these findings in human MSCs add to previous reports in other cell types that suggest a potential peripheral physiologic action of leptin. The positive local effects of leptin on bone metabolism could outweight leptin negative action exerted through the central nervous system pathway.

ACKNOWLEDGMENTS

The authors thank Dr. O. Brunser for critical review of the manuscript and valuable comments.

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