

In vivo leptin expression in cartilage and bone cells of growing rats and adult humans

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Abstract

The present investigation was carried out to analyse, immunohistochemically, *in vivo* leptin expression in cartilage and bone cells, the latter restricted to the elements of the osteogenic system (stromal cells, osteoblasts, osteocytes, bone lining cells). Observations were performed on the first lumbar vertebra, tibia and femur of four rats and on the humerus, femur and acromion of four patients. Histological sections of paraffin-embedded bone samples were immunostained using antibody to leptin. The results showed that, in growing rat bone, leptin is expressed in chondrocytes and stromal cells, but not in osteoblasts; bone lining cells were not found in the microscopic fields examined. In adult human bone, leptin is expressed in chondrocytes, stromal cells and bone lining cells; osteoblasts were not found in the microscopic fields examined. Osteocytes were found to be leptin positive only occasionally and focally in both rat and human bone. The *in vivo* findings reported show, for the first time, that leptin appears to be expressed only in the cells of the osteogenic lineage (stromal cells, bone lining cells, osteocytes) that, with respect to osteoblasts, are permanent and inactive, i.e. in those cells that according to our terminology constitute the bone basic cellular system (BBCS). Because the BBCS seems to be primarily involved in sensing and integrating mechanical strains and biochemical factors and then in triggering and driving bone formation and/or bone resorption, it appears that leptin seems to be mainly involved in modulating the initial phases of bone modelling and remodelling processes.

Key words bone; chondrocytes; immunohistochemistry; leptin; osteogenic cells.

Introduction

Leptin is a 16-kDa hormone, primarily secreted by adipose tissue, which controls body weight through its effects on food intake and energy expenditure by negative feedback at the hypothalamic nuclei (Zhang et al. 1994; Cinti et al. 1997; Ahima & Flier, 2000). Leptin is now known to have actions in the immune system (Lord et al. 1998), reproduction (Considine & Caro, 1999), development (Hoggard et al. 1997), haemopoiesis (Gainsford et al. 1996), angiogenesis (Sierra-Honigmann

et al. 1998) and, most recently, in bone metabolism. In fact two well-known clinical observations suggest that there is a link between bone mass, body weight and reproduction: obesity protects against bone loss, while menopause favours it (Riggs & Melton, 1986). However, there is considerable controversy surrounding the putative activity of leptin on bone. In *ob/ob*- and *db/db*-mice deficient, respectively, in leptin or its receptor, obesity and hypogonadism result in an increased bone mass (Ducy et al. 2000). With regard to the control of body weight, Ducy et al. (2000) hypothesized a central action for leptin and observed that leptin and its receptor did not appear to be expressed in osteoblasts. Takeda et al. (2002) have demonstrated that leptin's anorexigenic and anti-osteogenic effects are performed by two distinct neuronal pathways and that the sympathetic nervous system relays leptin's downstream signals via norepinephrine, which stimulates

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peripheral β_2 adrenergic receptors on osteoblasts, thus reducing bone formation. By contrast, Steppan et al. (2000) and more recently Yagasaki et al. (2003) have demonstrated that peripheral leptin administration has anabolic effects on bone metabolism in *ob/ob* mice: the demonstration that human osteoblasts *in vitro* express leptin receptor suggests that the bone growth-promoting effects of leptin could be direct. A leptin-positive effect on bone has also been suggested by Tamasi et al. (2003) in obese leptin receptor-deficient Zucker rats (*fa/fa*). In addition, Cornish et al. (2002) have demonstrated, in mice, that leptin tends to reduce bone fragility and that it also could contribute to the high bone mass and low fracture rates in obesity. In line with Steppan et al. (2000), other authors have demonstrated *in vivo* (Hoggard et al. 1997; Kume et al. 2002) and *in vitro* (Reseland et al. 2001; Cornish et al. 2002; Kume et al. 2002; Lee et al. 2002; Yagasaki et al. 2003) that leptin and its receptor are expressed on osteoblasts. More recently, Hamrick et al. (2004) have noted that in *ob/ob* mice the effects of altered leptin signalling on bone differs significantly in axial and appendicular regions of the skeleton.

Reports on the expression of leptin system in bone have mainly been provided by *in vitro* investigations, whose findings are often contradictory, as they did not allow permit conclusions to be made regarding whether leptin has a direct or indirect effect on osteoblasts. By contrast, very few *in vivo* studies have been conducted on the leptin system and they only concern murine fetal and neonatal bone (Hoggard et al. 1997; Kume et al. 2002). For this reason, the aim of the present immunohistochemical study under light microscopy (LM) was to analyse *in vivo* leptin expression in cartilage and in bone cells pertaining to the osteogenic lineage of growing rat and adult human bones.

Materials and methods

Animals

Observations were performed on the first lumbar vertebra, tibia and femur of four male Sprague–Dawley rats (from Charles River, Milan, Italy) aged 19 days.

Patients

Specimens of humerus, femur and acromion were obtained from four patients (two women, two men)

aged between 32 and 59 years. All patients underwent surgery for traumatic accident and authorized the biopsies for histological studies.

Antibodies used for immunohistochemistry

Rabbit polyclonal antibody raised against the peptide corresponding to amino acids 137–156 (COOH terminus) of the *ob* gene product (leptin) of human origin (A-20 lot. I 276; Santa Cruz Biotech, CA, USA) was used as primary antibody.

Immunohistochemistry

Tissue specimens from rat and human biopsies were fixed by immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS), pH 7.4. After washing in PBS, the samples were decalcified with EDTA 10%, dehydrated in a graded series of ethanol and embedded in paraffin (Paraplast). Sections (5 μm thick) were immunostained by means of the avidin–biotin technique (Hsu et al. 1981). They were incubated first with primary rabbit polyclonal anti-leptin antibody (Santa Cruz), diluted 1 : 40 in PBS, then with the corresponding biotinylated anti-rabbit IgG secondary antibody, made in goat, diluted 1 : 200 (Vector) and finally with ABC complex (Vectastain ABC kit, Vector). Peroxidase activity was revealed by diaminobenzidine hydrochloride as chromogen (Sigma, St Louis, MO, USA). Sections were then counterstained with haematoxylin, mounted in Eukitt (Kindler, Germany) and observed under LM (Axiophot, Zeiss).

Method specificity was tested (1) by omitting the primary antibody in the immunostaining procedures, and (2) by incubating the sections with the antiserum saturated with the homologous antigen. For the latter procedure, the antibody had been incubated with a 10-fold excess concentration of the homologous peptide (1 $\mu\text{g mL}^{-1}$) for 48 h (adsorption test).

Antibody specificity was tested by demonstrating leptin antigen in murine and human tissues: stomach was used as positive control and small intestine as negative control (Bado et al. 1998; Cinti et al. 2000). A further control experiment uncoupling protein-1 (UCP1), a specific marker for brown adipose tissue (Klaus et al. 1991), was performed both in bone samples (negative control) and in rat interscapular brown adipose tissue (positive control). A sheep anti-rat UCP serum (generously provided by Dr D. Ricquier, Meudon, France) was used at a final dilution of 1 : 4000.

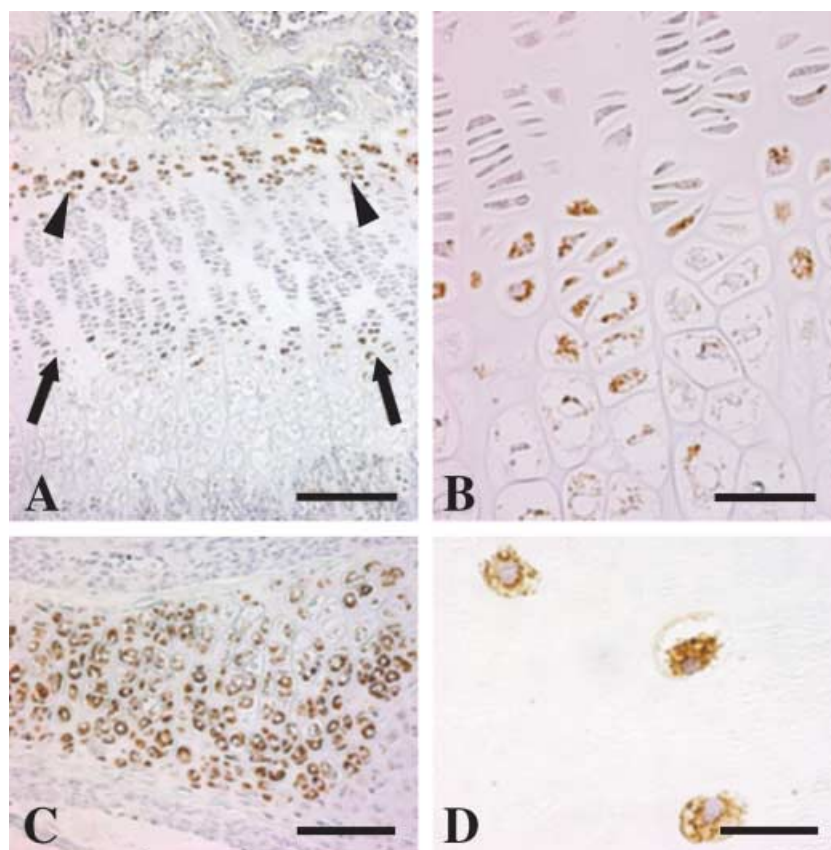


Fig. 1 Light micrographs showing immunoreactivity for leptin expression in chondrocytes of growing cartilaginous plates in proximal metaphysis of tibia (A,B) and in first lumbar vertebra (C) of a 19-day-old rat, and in cartilage of adult human acromion (D). Note in A that chondrocytes are leptin positive in the hypertrophic zone (arrows), enlarged in B, and in the layer facing the secondary ossification centre (arrowheads). Scale bars: A, 200 μm ; B, 50 μm ; C, 100 μm ; D, 20 μm .

Results

Rat bone

In 19-day-old rats, tibiae and femurs display both the diaphyseal primary ossification centre and the epiphyseal secondary centre. The body of the first lumbar vertebra is ossified in the central part, whereas its upper and lower surfaces are covered by growing cartilaginous plates.

Immunohistochemical analyses showed that leptin is expressed in both cartilage and bone cells. In metaphyseal growth plates, leptin is expressed in hypertrophic chondrocytes and in chondrocytes facing the secondary ossification centre (Fig. 1A,B). In addition, chondrocytes (Fig. 1C) in cartilaginous plates of the vertebral soma were found to be positive for leptin.

For bone, we only took into account the bone cells pertaining to the osteogenic lineage (stromal cells, osteoblasts, osteocytes, bone lining cells) in trabecular bone of both vertebral body and epiphyses as well as in cortical bone of the diaphysis of long bones. All bone surfaces observed appeared completely covered by osteoblast laminae in intense osteoformative activity; bone lining cells were never observed. Osteoblasts were found to be leptin negative, whereas bone

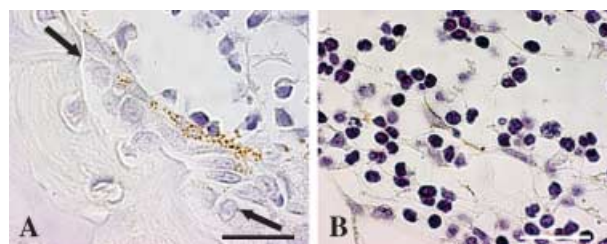


Fig. 2 Light micrographs showing stromal or haematopoietic cells positive for leptin expression in the marrow space of rat first lumbar vertebra (A) and of human femoral neck (B). Note in A the negative osteoblastic lamina (between arrows). Scale bars = 20 μm .

marrow stromal or haematopoietic cells as well as white adipocytes (positive internal control) were leptin positive (Figs 2A and 3A). Occasionally, osteocytes were found to be leptin positive, and in a focal manner (Fig. 4A). The negativity of vessels and blood cells constitutes a negative internal control.

Human bone

Immunohistochemical analyses performed in adult human bones showed that leptin is expressed in both

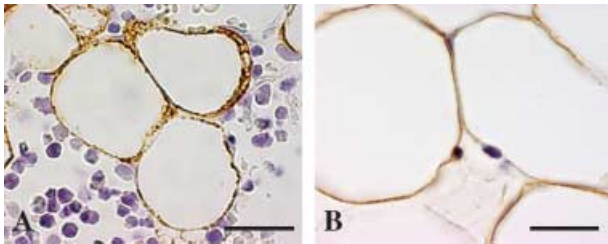


Fig. 3 Light micrographs showing adipocytes positive for leptin expression in the medullary space of rat tibia (A) and human humerus (B). Scale bars = 20 μm .

cartilage and bone cells, as in growing rat bone. Chondrocytes in the articular cartilage of the humeral head and acromion were positive for leptin (Fig. 1D).

For bone, no osteogenic surfaces were found, so we had no opportunity to analyse osteoblast immunoreactivity for leptin. The bone surfaces appeared to be in a resting phase and were covered by bone lining cells. Leptin expression was observed in bone lining cells (Fig. 5), bone marrow stromal or haematopoietic cells (Fig. 2B) and white adipocytes (positive internal control) (Fig. 3B). Osteocytes were occasionally leptin positive and in a focal manner (Fig. 4B,C), as in rat bone. The negativity of vessels and blood cells constitutes a negative internal control.

Controls

Leptin antibody stained gastric glandular epithelium (positive control), while all the cellular elements of small intestine were negative (negative control) in murine organs (data not shown). The addition of $1 \mu\text{g mL}^{-1}$ leptin in all the positive tests (adsorption test) gave negative results. Using UCP1 antibody, brown adipocytes of rat interscapular brown adipose tissue were positive, while bone cells and bone marrow cells (particularly white adipocytes and stromal or haematopoietic cells) were negative (data not shown).

Discussion

The present immunohistochemical study was performed

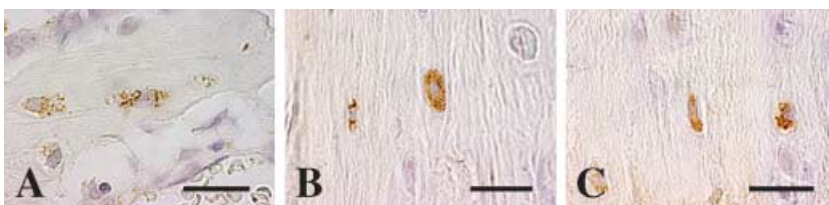


Fig. 4 Light micrographs showing some osteocytes positive for leptin expression in cortical bone of rat tibia (A) and in human acromion (B,C). Scale bars = 20 μm .

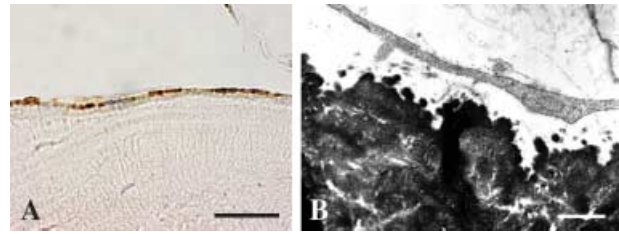


Fig. 5 Light micrograph (A) showing bone lining cells positive for leptin expression, covering the bone surface in a resting phase, in the proximal end of adult human humerus. A transmission electron micrograph (B) demonstrates that the brownish strip bordering the bone surface in A really corresponds to bone lining cells. Scale bars: A, 20 μm ; B, 0.5 μm .

to analyse *in vivo* leptin expression in chondrocytes and in bone cells pertaining to the osteogenic lineage.

The positivity for leptin expression we found in chondrocytes appears to be in line with *in vivo* and *in vitro* findings recorded by other authors (Kume et al. 2002). Note that chondrocytes have also been found to express leptin receptor (Steppan et al. 2000; Figenschau et al. 2001; Cornish et al. 2002; Kume et al. 2002; Gat-Yablonski et al. 2004). Because leptin has been observed to be present in synovial fluid and in chondrocytes of articular cartilage of patients affected by osteoarthritis as well as in osteophytes, it has been postulated that leptin might exert a modulatory effect on chondrocyte metabolism and consequently on the pathogenesis of osteoarthritis (Dumond et al. 2003).

Our results on leptin expression in bone marrow adipocytes are also in agreement with those obtained from primary culture of human bone marrow adipocytes (Laharrague et al. 1998). Because such adipocytes are close to marrow stromal cells and to the other cells of the osteogenic lineage, we agree with the hypothesis suggested by other authors that adipocyte-derived leptin, in addition to its well-established endocrine role, could serve as a paracrine factor in modulating the activity of the osteogenic cells and the differentiation of haematopoietic precursor cells (Bennet et al. 1996; Gainsford et al. 1996).

In vitro findings on leptin expression appear to be contradictory: according to Ducy et al. (2000) rat osteoblasts are leptin negative, whereas according to Reseland et al. (2001) they are leptin positive. Because the so-called osteoblasts used for *in vitro* studies are in fact osteoblast-like cells, which are identifiable only on the basis of some specific markers, we do not think that our *in vivo* results can be compared with those from *in vitro* investigations. In fact osteoblast-like cells include both osteogenic-active elements (osteoblasts) and osteogenic-inactive elements (osteocytes, bone lining cells, stromal cells), which we found in our *in vivo* study to be negative and positive, respectively, for leptin expression. Indeed, Hoggard et al. (1997) found *in vivo* positivity for leptin in fetal cartilage/bone, but failed to attribute leptin expression to a particular differentiated cell type.

The fact that we found all cells of the osteogenic lineage, excluding osteoblasts, to be positive for leptin expression leads us to hypothesize that the expression of this protein might occur in inverse fashion to osteogenic activity. It should be noted in this connection that a similar pattern has been recently demonstrated in adipocytes: leptin was found to be expressed in unilocular brown adipocytes (cells in inactive thermogenic phase) and unexpressed in multilocular brown adipocytes (cells in active thermogenic phase) (Cancello et al. 1998).

The above inverse leptin expression behaviour, as regards bone formation activity, mirrors our recent morphofunctional studies on the cells of the osteogenic lineage. We found that such cells constitute a continuous cytoplasmic network extending from the vascular endothelium to the osteocytes, passing through stromal cells, bone lining cells or osteoblasts (according to whether the surface is resting or growing, respectively). As gap junctions (electrical synapses) connect all these cells, they have been considered a functional syncytium, along which mechanical and metabolic signals can be issued by wiring and volume transmission (Marotti, 1996, 2000; Palazzini et al. 1998; Palumbo et al. 2001; Rubinacci et al. 2002). Note that, of the cells of the osteogenic lineage, osteoblasts are transient elements which appear during bone formation only, whereas the other cells of the osteogenic lineage (stromal cells, bone lining cells, osteocytes), unlike osteoblasts, are permanent elements and in an inactive phase. For this reason we suggested that the cells of the osteogenic lineage, excluding osteoblasts, form a

sort of bone basic cellular system (BBCS) capable of sensing and integrating mechanical strains and biochemical factors and then triggering bone formation or bone resorption. The fact that leptin expression is positive on the cells of the BBCS (though occasionally and focally on osteocytes) suggests the hypothesis that such protein could mainly play a role in modulating BBCS activity.

We are aware that the leptin effect, not only on bone metabolism but also over a larger extent, remains controversial, notwithstanding the huge amount of data recorded. In particular, the effect of leptin on bone metabolism might be dependent on concentration and mode of exposure, determined by the source, from which leptin reaches bone cells. A central negative effect (Ducy et al. 2000) could counterbalance that peripheral positive effect (Steppan et al. 2000), the latter being predominant in central leptin resistance occurring during obesity, or when there are high serum leptin levels, a signal of elevated energetic storage. This is supported by a recent study demonstrating a correlation between serum leptin levels and bone mineral density only when leptin values were higher than the threshold of leptin resistance occurrence (Ghazali et al. 2003). Finally, we suggest a paracrine way of leptin action on bone derived from the bone itself and/or bone marrow adipocytes that undoubtedly requires further morphofunctional investigations.

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