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Potential Induction of Osteogenesis by Systemic Administration of Bovine Bone Proteins

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This study shows, apparently for the first time, that the administration of bone derived proteins (putative bone growth factors) obtained from bovine demineralized maxillaries has a direct effect on osteogenesis, affecting significantly some markers of bone formation such as lactate dehydrogenase activity and serum osteocalcin. Also, collagen deposition and bone protein turnover were markedly increased by the treatment, which may have important biological and clinical applications.

Key Words: Bone matrix, Osteogenesis, Bone growth factors.

Bone is a tissue that is continuously renewed during postnatal life (7). The extraordinary capacity for growth, remodelling and regeneration of bone has been mainly attributed to the proliferation of predifferentiated osteoprogenitor cells and the induction of the differentiation of mesenchymal type cells into cartilage and bone (14).

In this context, bone formation is a complex process which is not only controlled by hormones (44) or nutritional status (20), but also by systemic growth factors (EGF, FGF, PDGF, IGF, etc.) acting on specific bone target cells and local growth factors through autocrine and paracrine mechanisms (6).

These factors, generally referred to as bone derived growth factors or BDGF'S (37), apparently may induce changes in bone cell replication and on differentiated function, which are primarily represented by changes in bone collagen synthesis. The role of local growth factors occurring in the extracellular bone matrix has not been fully characterized (9).

Biological activities of BDGF'S have been estimated to date by using implant models (24, 34) or bone cells cultures (2), hence this experiment was conducted in order to evaluate the possible effects of subcutaneously injected bone matrix pro-

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teins on bone composition, growth and metabolism.

Materials and Methods

Bone-inductive preparation. — Freshly excised maxillary bone of young bovine animals were carefully scraped, cleaned off soft tissue and thoroughly washed with distilled water and later dried at 38 °C until constant weight (maxillary weight about 1.5 kg). Then, bones were broken into fragments and pulverized in a hammer mill to particle sizes ranging from 74 to 420 µm. The bone powder was defatted with ethylether (1:2 v/v) during 60 min, under continuous stirring. The resulting dried powder was demineralized in 0.6 N HCl at 2-4 °C (25 mmol/l) during 24 hours. The acid-insoluble precipitate was repeteadly washed in cold distilled water and later lyophilized before subcutaneous administration.

The bone extract of bovine maxillaries reached a 94 % of protein (initial about 23 %) while the mineral content was 3 % (initial about 70 %). The analysis of the constituents of the bone extracts were carried out according to the AOAC methods (1).

Bioassay. — Rats of the Wistar strain with initial weights about 100 g, received twice daily (09:00 and 20:30 h) a subcutaneous injection of the decalcified bone matrix (0.01 g/kg BW) in ClNa 0.9 % as vehicle, while controls were administered with saline.

During the experimental period of 41 days, body weight was measured daily. Also, femur, tibia, gastrocnemius muscle and liver of rats were carefully excised and weighted after the animals were killed. Tibia and femur bones of both legs were frozen prior to analysis.

Femur bone composition (moisture and minerals) were determined by standard analytical procedures (1). Bone hydroxyproline was determined according to WOESSNER (41), as indicator of collagen content (% hydroxyproline × 7.14 = % collagen).

Femur alkaline phosphatase activity, as index of bone formation (10), and tibia lactate and malate dehydrogenase, as indicators of bone cellular metabolism were also determined (33).

Moreover, the serum and urinary calcium and phosphorus contents were measured by atomic absorption spectophotometry to complete the study about bone mineral metabolism, as described elsewhere (4).

Serum osteocalcin, other marker of osteogenic induction, was determined by a radioimmunoassay procedure, as previously reported (12).

Finally, bone protein synthesis (Ks, %/day) was performed by using the phenylalanine flooding dose method as validated for intraperitoneal injection (25), which has been specifically used in the measurement of this tissue (30). Briefly, radiolabelled phenylalanine (50 µCi/ml) was combined with unlabelled phenylalanine (150 mM) and the rats were injected with 1 ml/100 g BW. Ten minutes after administration, the rats were decapitated and the bones were carefully excised, washed in saline and blotted prior to freezing in liquid nitrogen. The fractional synthetic rate of proteins (Ks, %/day) was calculated as follows:

$Ks = (Sb \times 100)/(Si \times t)$, where: Sb = specific activity of phenylalanine in protein (dpm/µmol); Si = specific activity of tissue-free phenylalanine (dpm/µmol); t = time of labelling (aprox 10 min).

Specific activities were estimated by fluorometric measurements and scintillation counting (25, 30).

Statistical analysis. — The Student «t» test was used to evaluate statistical differences between both groups (36). A value of $P < 0.05$ was considered significant.

Results

Bone extract obtained from bovine maxillaries contains a high proportion of proteins (94.0 %) and low levels of water (2.0 %), ash (3.1 %) and fat (1.2 %). Other components analyzed on bone extracts were hexosamines (0.1 %), phosphorus (0.4 %) and DNA (< 1 %) and RNA (< 1 %). A more detailed characterization of bone extract shows that its principal content is collagen (74.9 %) with very low levels of calcium (0.2 %).

No changes in bone, gastrocnemius muscle, liver and final body weights were observed after the subcutaneous injection of bovine demineralized bone matrix to rats. While an increase in collagen deposition as assessed by the rise in bone concentration of hydroxyproline was noted, no changes in other constituents were detected (table I).

Also, the process of bone formation assessed by bone alkaline phosphatase (AP), lactate and malate dehydrogenase activities (LDH and MDH) increased in those animals subcutaneously administered the bone derived proteins (fig. 1).

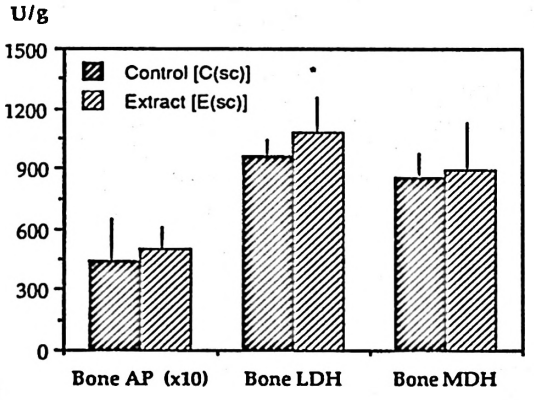

Fig. 1 *Femur alkaline phosphatase activity (AP) and tibia lactate and malate dehydrogenase activities (LDH and MDH) of subcutaneous administered animals with demineralized bone matrix [E(sc)] and control animals [C(sc)].*
The rectangles and bars represent the mean values and standard deviations (n = 8; *p < 0.05).

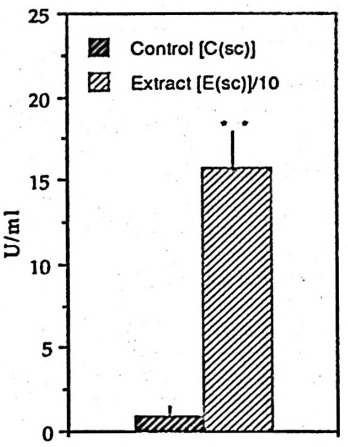

Fig. 2 *Serum osteocalcin concentration of subcutaneously administered rats with demineralized bone matrix [E(sc)] and control rats [C(sc)].*
The rectangles and bars represent the mean values and standard deviations (n = 8; **p < 0.01).

Table I. *Bone (femur) water, mineral, hydroxyproline and collagen contents of subcutaneously administered animals with demineralized bone matrix [E (sc)] and control animals [C (sc)]. (Means ± SD; n = 8).*

Variables (%)	Control [C(sc)]	Extract [E(sc)]	Statistical analysis
Water	33.62 ± 1.78	33.03 ± 2.04	NS
Mineral salts	57.89 ± 2.50	58.39 ± 1.52	NS
Hidroxyproline	1.84 ± 0.21	2.07 ± 0.25	p < 0.06
Collagen	13.18 ± 1.55	14.78 ± 1.80	p < 0.06

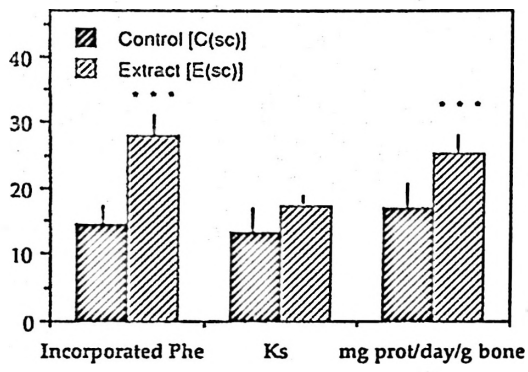

Fig. 3 *Relative incorporation of phenylalanine to bone (tibia) protein (%/day), daily protein synthesis rates, Ks, (%/day) in bone (tibia) and absolute protein synthesized by day and gram of bone (mg protein/day/g bone) of s.c. administered animals with demineralized bone matrix [E(sc)] and control animals [C(sc)].*
The rectangles and bars represent the mean values and standard deviations (n = 8; ***p < 0.001).

Serum osteocalcin levels, measured as a specific marker of the osteogenic potential of the demineralized bone matrix, significantly increased (fig. 2) as well as the values of phenylalanine incorporation into protein (%/day) and daily bone protein synthesis (mg protein synthesized/day) in the matrix treated animals as compared with controls (fig. 3).

Discussion

Xenogeneic bone formation in rats at extraskeletal sites has been repeteadly reported after subcutaneous implantation of bone preparations (3, 38) as well as with this bovine demineralized bone matrix (24).

Some of these bone preparations containing local bone growth factors, induce bone formation in cell cultures (15), while some actions on bone metabolism have been observed with other growth factor, such as human skeletal growth factor (13), fibroblastic growth factor (15), epidermal growth factor (38) or insulin-like growth factors (22), and also by exogenous administration of growth hormone to intact rats (26).

Many investigators have tried to identify different growth factors occurring in bone matrix with osteogenic activity (5, 43), which is very important to understand the bone metabolism and also for clinical applications concerning the replacement of cartilage and bone (40, 45). Recent observations suggest that most of the active compound inducing bone formation are polypeptides or glycoproteins (5). Some homologies between inductive proteins from different species have also been reported (35).

Thus, recently some authors have identified different osteogenic proteins (bone morphogenetic proteins or BMP) that irreversibly induce differentiation of perivascular mesenchimal cells into osteoprogenitor cells (18), while several other bone matrix constituents elaborated for and by bone cells would increase the proliferation of osteogenic precursors of the synthesis capacity of differentiated osteoblast (43), such as osteogenin (39), osteoinductor factor (5), transforming growth factor β and microglobulin β_2 (8).

Currently, bone formation and regeneration are assumed to be attributable to the coefficiency of various bone derived growth factors (38). Other non-collagenous proteins such as osteonectin and osteocalcin have a role in mineralization, but as yet, a definitive effect on bone induction has not been established (29). Finally, other agents such as prostaglandins, fluoride and different peptides released by satellite cells could participate in bone accretion and resorption (19, 22).

In this context, this experiment evaluates, apparently for the first time, the effects of the subcutaneous administration of bone preparations of xenogeneic origin on rat bone metabolism, which have been repeteadly studied as osteogenic inducers

by using implant models (24, 34) or bone cultures (2).

Our experimental results suggest that subcutaneous administration of demineralized bone matrix to rats induces new bone formation, favouring the ossification process in bones and increasing osteoblastic activity. Thus, a remarkable increase in bone hydroxyproline and collagen content ($p < 0.06$), serum osteocalcin levels ($p < 0.01$) and phenylalanine incorporation into protein ($p < 0.001$) has been observed in the subcutaneously treated animals with the demineralized bone matrix as compared to the controls.

Long bones are easily removed and scraped and contain large amounts of extracellular matrix (26). Moreover, most authors have used these bones as the source of bone extract (30, 33).

This study has been carried out in male growing rats, because this animal model has previously been used in trials concerning bone growth (24) with different anabolic agents (28). Also, protein turnover in young animals is more sensitive to growth and skeletal promoters (26, 27).

Serum osteocalcin levels, a bone specific protein, increased in a situation of bone formation (42), and its activity is related to alkaline phosphatase activity (21).

These statements, postulated by different authors (10, 29), confirm the results obtained in our experimental trial, since the administration of demineralized bovine maxillar extract, produced a significant increase, even in femur hydroxyproline and collagen content of treated animals as well as in serum osteocalcin levels as compared with controls. Moreover, the serum osteocalcin levels increased in all treated animals (100 %) with the demineralized bone extract.

Serum osteocalcin levels are used in clinical measurements as a specific biochemical index because it is an early marker of osteoblastic activity, which has beenused for diagnostic purposes (32). Furthermore, a correlation between certain nutritional or hormonal profiles and serum osteocalcin levels (16) has been found. Thus, a decrease in serum osteocalcin concentration is associated with a lower bone density (23).

The values of protein synthesis appear to indicate that the subcutaneous administration of demineralized bone matrix to rats, induces new bone protein formation, altering the protein turnover. This increase in protein synthesis agrees with the enhancement in hydroxyproline and collagen content in extract treated rats as well as in serum osteocalcin levels.

Despite the fact that bones are only a small component of the rat weight, they account for approximately 8 % of whole-body protein synthesis (30). The measurement of the rates of protein synthesis in skin and bone, their contribution to whole-body protein metabolism, and their response to nutritional stress, have been previously estimated by using the phenylalanine flooding as method (26, 30). This method uses a large dose of labelled phenylalanine to minimize the difference between tissue and plasma specific radioactivities during the period of incorporation of label into protein, which occurs in quantitative proportions in bone used for protein synthesis measurements (31).

In recent studies concerning muscle protein metabolism (11, 27) and skin and bone protein synthesis of young rats (30), greater values than ours have been reported, using the same experimental procedure, which could be due to differences in the administration route of phenylalanine as well as the age of the experimentation animals or the bone fraction analyzed, which is directly related to the metabolic kinetics of each rat.

Furthermore, all these results are in good agreement with those obtained through enzyme determinations. Thus, a marked increase in bone alkaline phosphatase activity has been observed, as specific index of new bone formation, and in

the specific catalytic enzyme activities, LDH and MDH, of treated rat bones as compared to the controls, which suggests the involvement of different mechanisms at the cellular level. Nevertheless, the bone mineralization process of animals treated with bovine matrix extracts, remains apparently unaltered as compared with controls, and as determined by the mineral composition of rat bones and the calcium and phosphorus content in serum and urine. Some investigators in very preliminary studies have determined the calcium and phosphorus concentration in serum and urine of implanted rats with demineralized bone extracts, obtaining similar results to those of this experimental assay (17).

The biological activity of extracellular bone matrix extract could be affected by sex, strain, age, nutritional status, and by pattern of treatment (dose, time, course of administration, etc.) (9).

Summing up, our experimental results describe an osteogenic potential of demineralized bone matrix when it is administered subcutaneously to rats, affecting bone protein apparently more than mineral constituents. Further studies are needed in order to optimize the utilization of those bone derived proteins for molecular biology purposes or for clinical applications (40).

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Resumen

Se muestra, posiblemente por vez primera, que la administración subcutánea de proteínas derivadas del hueso (factores de crecimiento putativos), obtenidas a partir de maxilares desmineralizados de bovino, tiene un efecto directo sobre la osteogénesis modificando algunos marcadores de la formación ósea, tales como la actividad lactato deshidrogenasa y los niveles de osteocalcina sérica. También aumentan significativamente, como consecuencia del tratamiento, el depósito de colágeno y la síntesis de proteína en hueso, lo cual puede conllevar importantes aplicaciones biológicas y clínicas.

Palabras clave: Matriz ósea, Osteogénesis, Factores de crecimiento óseos.

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