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Lactoferrin promotes bone growth

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Abstract

We have demonstrated bovine or human lactoferrin to be an anabolic factor in skeletal tissue. *In vitro*, lactoferrin stimulates the proliferation of bone forming cells, osteoblasts, and cartilage cells at physiological concentrations (above 0.1 μg/ml). The magnitude of this effect exceeds that observed in response to other skeletal growth factors such as IGF-1 and TGFβ. DNA synthesis is also stimulated in a bone organ culture system likely reflecting the proliferation of cells of the osteoblast lineage. Lactoferrin is also a potent osteoblast survival factor. In TUNEL and DNA fragmentation assays, lactoferrin decreased apoptosis, induced by serum withdrawal, by up to 70%. In addition, lactoferrin has powerful effects on bone resorbing cells, osteoclasts, decreasing osteoclast development at concentrations >1 μg/ml in a murine bone marrow culture system. However, lactoferrin did not alter bone resorption in calvarial organ culture, suggesting that it does not influence mature osteoclast function. *In vivo*, local injection of lactoferrin in adult mice resulted in increased calvarial bone growth, with significant increases in bone area and dynamic histomorphometric indices of bone formation after only 5 injections. Taken together, these data demonstrate that the naturally-occurring glycoprotein lactoferrin is anabolic to bone *in vivo*, an effect which is consequent upon its potent proliferative and anti-apoptotic actions in osteoblasts, and its ability to inhibit osteoclastogenesis. Lactoferrin may therefore have a physiological role in bone growth, and a potential therapeutic role in osteoporosis.

Introduction

Bone growth is controlled by many hormones and growth factors and bone is continually remodelled by the complex coupling of the actions of the bone forming cells, osteoblasts, and bone resorbing cells, the osteoclasts (Mundy *et al.* 1999a) Milk is a rich biological fluid, which functions to provide nutrition at a time of very rapid skeletal growth and development in the neonate. Because of this, it contains growth regulators, in addition to the simple substrates necessary for infant development (Lonnerdal & Iyer 1995). Therefore, we have assessed the effects of milk proteins on bone cell growth, and have found that a number of fractions of whey protein have growth stimulatory effects in primary cultures of osteoblasts. With a view to determining the identity of the growth promoting molecules within whey protein, we used HPLC to identify the major proteins in the active fractions. We found that the glycoprotein, lactoferrin, was present in many of these fractions. On this basis, we hypothesized that lactoferrin stimulates osteoblast growth.

The present studies address the bone actions of lactoferrin using assessments of osteoblast growth and survival, osteoclast development and activity, its actions on intact bone tissue *in vitro*, and in intact animals. These studies establish lactoferrin as a potent novel anabolic factor in osteoblasts, which also reduces bone resorption. These findings pose important questions regarding its role in normal bone physiology both during growth and in adulthood, and provide a novel mechanistic pathway as a target for drug development in the therapeutics of osteoporosis.

Material and methods

Lactoferrin

Lactoferrin was isolated from fresh bovine skim milk by cation exchange chromatography and gel filtration. Briefly, the milk was passed through S Sepharose Fast Flow at 4C and the bound proteins eluted in steps with 0.1 M, 0.35 M and 1 M NaCl. The 1 M NaCl fraction containing lactoferrin was dialysed and freeze-dried. The material was then dissolved in 25 mM sodium phosphate buffer pH 6.5 and reapplied to the cation exchanger, which had been equilibrated in the above buffer. Lactoferrin was eluted by application of a salt gradient to 1 M NaCl in phosphate buffer and the recovered material dialysed and freeze-dried. Final purification of lactoferrin was achieved by gel filtration through Sephacryl S300 in phosphate buffer and the protein recovered as a dialysed freeze-dried powder.

Osteoblast cultures

Primary cultures of fetal rat osteoblasts and human osteoblasts were used to assess the effects of lactoferrin on osteoblast-like cells. Primary rat osteoblast cells were derived from sequential collagenase digestion of 21-day fetal rat calvariae (Cornish *et al.* 1999). Human osteoblasts were grown from enzyme-treated trabecular bone chips collected at surgery (Robey & Termine 1985). Cell growth was monitored by measurements of thymidine incorporation and cell number. Osteoblast apoptosis was assessed using either the TUNEL method (using a kit: DeadEnd^{TM}, Promega, Madison, WI) or DNA fragmentation assays (Grey *et al.* 2002). These osteoblast cultures were also prolonged, permitting observation of the development of a mature phenotype through the formation of bone nodules. These cultures required the presence of 50 μg/ml ascorbic acid-2-phosphate and 10 mM sodium β-glycerophosphate. Media was changed every third day. Lactoferrin was replaced with every media change. After 21 days the cells were fixed in neutral buffered formalin and stained for mineralisation by the von Kossa method. The area of bone nodules was quantified using image analysis.

Chondrocyte cultures

Because many factors that influence osteoblast growth have similar effects on chondrocytes, we also assessed these factors in chondrocyte cultures. These cells were prepared by removing cartilage (full-depth slices) from the tibial and femoral surfaces of sheep under aseptic conditions (Cornish *et al.* 1999). Measurements of thymidine incorporation was performed in growth-arrested cell populations as for the osteoblast-like cell cultures.

Bone organ culture

Cultures of neonatal mouse calvariae were utilised as a model of mature osteoclast function (there is virtually no osteoclast development in this model because of the absence of bone marrow in the calvariae) (Cornish *et al.* 1999). The calvariae also provided a second model in which osteoblast function was studied.

Isolated osteoclasts

Mature osteoclasts cultured on devitalised bone chips were isolated from long bones of 1-day old rats (Cornish *et al.* 2001). The amount of bone resorbed was measured by reflective microscopy and image analysis.

Bone marrow cultures from mice were used to assess the impact of these factors on osteoclast development (Cornish *et al.* 2001). After culturing for 7 days, adherent cells in the tissue culture plates were fixed with citrate-acetone-formaldehyde and stained for tartrate-resistant acid phosphatase (TRAP) using SIGMA Kit #387-A. TRAP-positive multi-nucleated cells were counted in all wells.

In vivo *studies*

Sub-acute effects of lactoferrin were assessed in a model where lactoferrin or vehicle were given as daily injections over the periosteum of the right hemicalvaria of normal adult mice over a period of 5 days. The animals were sacrificed 1 week following the last injection. We have found the results obtained in this model to be in good agreement with those derived from longer-term studies of the systemic administration of peptides (Cornish *et al.* 2000).

Results

Lactoferrin stimulates osteoblast proliferation and differentiation

Lactoferrin produced a dose-related increase of thymidine incorporation in primary cultures of rat and

human osteoblast-like cells at 24 h. This effect was present at concentrations of lactoferrin that occur *in vivo* (1–100 μg/mL). The above studies of lactoferrin action on osteoblast proliferation were complemented by an assessment of its action on differentiation of these cells. These studies were performed using 3-week cultures of primary rat osteoblasts to assess bone nodule formation, involving bone matrix deposition and mineralisation. Lactoferrin, at a concentration of 100 μg/mL, significantly stimulated the number and area of bone nodules formed.

Lactoferrin acts as a potent survival factor

Lactoferrin decreased apoptosis observed in response to serum deprivation in cultures of primary rat osteoblastic cells, as judged by the number of TUNEL-positive cells. This effect was dose-dependent and apparent at similar concentrations to those causing osteoblast proliferation. The anti-apoptotic effect of lactoferrin in osteoblastic cells was confirmed in these cells using a DNA fragmentation assay. In these assays, lactoferrin reduced the osteoblast apoptosis significantly, indicating that lactoferrin is a potent survival factor in these cells.

Lactoferrin stimulates chondrocyte proliferation

In view of the effects of lactoferrin on osteoblasts, its effect on proliferation of primary cultures of ovine chondrocytes was also assessed. At concentrations of 10 and 20 μg/mL, lactoferrin induced statistically significant increases of thymidine incorporation into ovine chondrocytes, which were at least as great as those seen in osteoblasts.

Lactoferrin potently inhibits osteoclastogenesis

The effects of lactoferrin on osteoclast development were assessed in mouse bone-marrow cultures. The number of newly developed osteoclasts, assessed as multinucleated cells staining positively for TRAP, was significantly decreased by lactoferrin in concentrations of 10 μg/mL and greater. At 100 μg/mL, osteoclastogenesis was completely arrested.

Lactoferrin has no effect on mature osteoclast activity

In contrast to the observed inhibitory effect on osteoclast development, lactoferrin had no effect on bone resorption by isolated mature osteoclasts. Results in neonatal mouse calvariae were consistent with those from isolated cell cultures. Lactoferrin increased thymidine incorporation in the calvarial organ culture, reflecting the proliferation of cells of the osteoblast lineage. However, bone resorption measured as calcium release from cultured neonatal mouse calvariae was unaffected. Because there is virtually no bone marrow in this tissue, these explants reflect mature osteoclast function. Thus, this finding is consistent with that in isolated mature osteoclasts.

Lactoferrin increases bone growth in vivo

Four groups of sexually mature male mice were given daily injections of one of 3 doses of bovine lactoferrin (0.04 mg, 0.4 mg or 4 mg) or vehicle over the right hemicalvaria for 5 consecutive days. Both 0.4 mg and 4 mg of lactoferrin produced increases in new bone formation, the latter being four-fold greater than control (Figure 1). Dynamic histomorphometric indices of bone formation were also assessed and lactoferrin increased the extent of the double-labelled surface as well as the mineralising surface in a dose-dependent fashion.

Discussion

This study demonstrates that lactoferrin is a promoter of osteoblast growth. Its effects on both the proliferation of osteoblasts (assessed by thymidine incorporation and by cell numbers) and in the prevention of osteoblast apoptosis are profound, being far greater than classic osteoblast growth factors which we have studied in these models, such as IGF-1 or TGF-β. This growth stimulating potency is complemented by its capacity to virtually halt osteoblast apoptosis. Again, this is much more dramatic than the effects that we have seen with other factors, e.g. IGF-1. Lactoferrin's effects on cell numbers are complemented by the growth stimulation of a more differentiated osteoblast phenotype, thus promoting bone matrix deposition and mineralisation. The degree of iron saturation of the bovine lactoferrin preparations does not appear to greatly influence the osteogenic behaviour of the glycoprotein, in that apo and holo preparations have similar potency to the native preparations of approximately 20% iron saturation (Cornish *et al.* manuscript in preparation). The actions of lactoferrin on bone resorption are quite the reverse, in that it produces an almost total arrest of osteoclastogenesis in mouse bone marrow cultures. Even though it does not influence

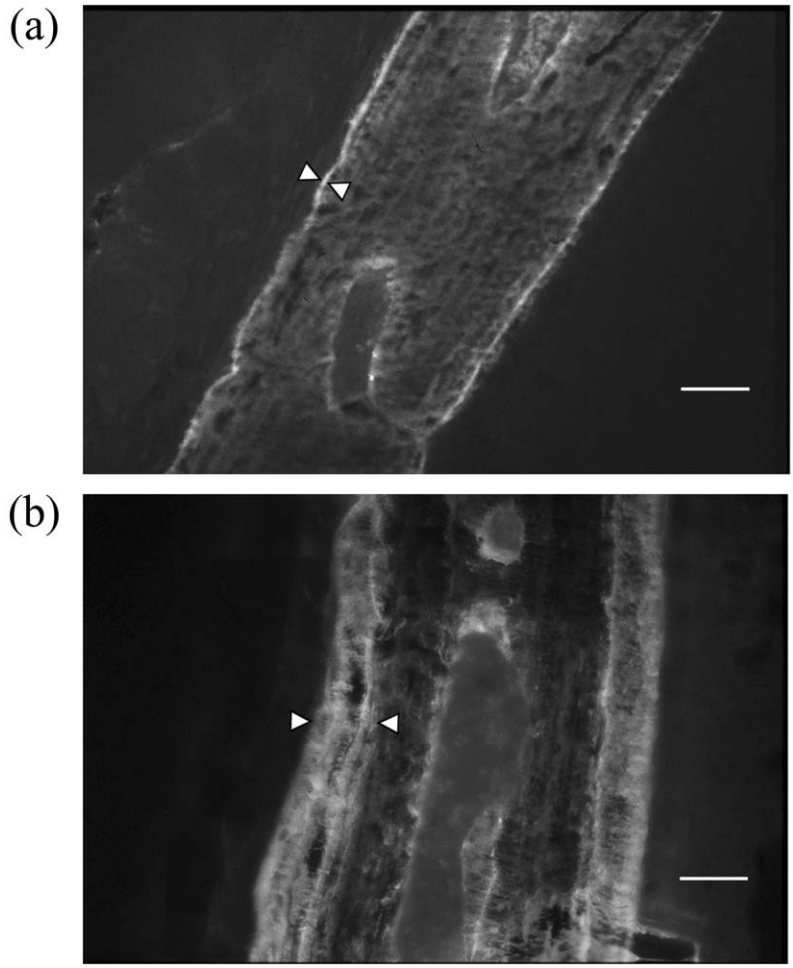

Fig. 1. Photomicrographs of calvarial bones taken from mice treated with (a) vehicle and (b) lactoferrin (4 mg), following a short term *in vivo* study in which the factors are administered subcutaneously above the skull bone. The amount of bone formed during a 13-day period is indicated between the 2 arrow-heads and dramatic increases in bone mass can be seen in the lactoferrin-treated bone compared to control. Bar = 50 microns.

the activity of mature osteoclasts, this is still likely to result in a profound reduction in bone resorption activity. The bone effects of lactoferrin only appear to have been assessed by one other group. Lorget *et al.* (2002) have demonstrated that bovine lactoferrin is able to reduce bone-resorbing activity in a rabbit mixed bone cell culture, which is broadly consistent with the present findings.

As a result of these combined effects on bone formation and bone resorption, even the very short term *in vivo* exposure to lactoferrin studied here, produces dramatic increases in bone mass. The lactoferrin effects appear comparable to the stimulation of bone formation demonstrated by Mundy *et al.*, following local injection of statins (Mundy *et al.* 1999b). Thus, lactoferrin appears to be among the most potent known regulators of bone mass. This potency is further attested to by the increases in new bone formation seen at sites remote from the injection site there is evidence of increased formation on the intracranial aspect of the calvariae and on the contralateral side (data not shown), something we have not seen with most other agents studied in this model.

Taken together, these data demonstrate that the naturally-occurring glycoprotein, lactoferrin, is anabolic to bone *in vivo*, an effect which is consequent upon its potent proliferative and anti-apoptotic actions in osteoblasts, and its ability to inhibit osteoclastogenesis. Lactoferrin may therefore have a physiological role in bone growth, as well as a potential therapeutic

role in osteoporosis or for local use in orthopaedic practice to promote the healing of fractures or filling in of bone defects.

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