Lactoferrin Is a Potent Regulator of Bone Cell Activity and Increases Bone Formation in Vivo

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Lactoferrin is an iron-binding glycoprotein present in epithelial secretions, such as milk, and in the secondary granules of neutrophils. We found it to be present in fractions of milk protein that stimulated osteoblast growth, so we assessed its effects on bone cell function. Lactoferrin produced large, dose-related increases in thymidine incorporation in primary or cell line cultures of human or rat osteoblast-like cells, at physiological concentrations (1–100 μg/ml). Maximal stimulation was 5-fold above control. Lactoferrin also increased osteoblast differentiation and reduced osteoblast apoptosis by up to 50–70%. Similarly, lactoferrin stimulated proliferation of primary chondrocytes. Purified, recombinant, human, or bovine lactoferrins had similar potencies. In mouse bone marrow cultures, osteoclastogenesis was dose-dependently decreased and was completely arrested by lactoferrin, 100 μg/ml, associated with decreased expression of receptor activator of nuclear factor-κB ligand. In contrast, lactoferrin had no effect on bone resorption by isolated mature osteoclasts. Lactoferrin was administered over calvariae of adult mice for 5 d. New bone formation, assessed using fluorochrome labels, was increased 4-fold by a 4-mg dose of lactoferrin. Thus, lactoferrin has powerful anabolic, differentiating, and antiapoptotic effects on osteoblasts and inhibits osteoclastogenesis. Lactoferrin is a potential therapeutic target in bone disorders such as osteoporosis and is possibly an important physiological regulator of bone growth. (Endocrinology 145: 4366–4374, 2004)

Milk is a rich biological fluid that functions to provide nutrition at a time of very rapid skeletal growth and development in the neonate. Because of this, it contains many growth regulators in addition to the simple substrates necessary for infant development. Therefore, we assessed the effects of milk proteins on bone cell growth, and 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.

Lactoferrin is an 80-kDa iron-binding glycoprotein that belongs to the transferrin family of proteins (1). It is produced by many exocrine glands and, consequently, is widely distributed in body fluids including tears, saliva, bile, pancreatic fluid, vaginal secretions, semen, and milk (2). Lactoferrin is also a major constituent of the secondary granules of neutrophilic leukocytes, from which it is released during acute inflammation (3). Serum levels of lactoferrin in healthy subjects range from 2 to 7 μg/ml and are predominantly neutrophil derived, but during inflammation and sepsis its local concentrations may be much higher (4). It acts as an iron chelator, which may contribute to its antimicrobial activity (5), but it also has effects on cell growth and differentiation (6), embryonic development (7), myelopoiesis (8), endothelial cell adhesion (9), cytokine (10, 11) and chemokine (12) production, regulation of the immune system (13), and modulation of the inflammatory response (14). Its effects on bone have received little attention.

The present studies address the skeletal effects of lactoferrin in vitro (using assays of osteoblast growth, differentiation and survival, osteoclast development and activity, and bone organ culture) and in vivo. These studies establish lactoferrin as a potent novel anabolic factor in osteoblasts, which also reduces bone resorption and increases bone mass when administered in vivo. These findings pose important questions regarding the role of lactoferrin in bone physiology, both during growth and in adulthood, and provide a potential target for drug development in the therapeutics of osteoporosis.

Materials and Methods

Osteoblast-like cell culture

Osteoblasts were isolated from 20-d fetal rat calvariae, as previously described (15). Briefly, calvariae were excised and the frontal and parietal bones, free of suture and periosteal tissue, were collected. The calvariae were sequentially digested using collagenase and the cells

Abbreviations: FBS, Fetal bovine serum; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; TRAP, tartrate-resistant acid phosphatase; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.
from digests 3 and 4 were collected, pooled, and washed. Cells were grown in T75 flasks in 10% fetal bovine serum (FBS) (Gibco BRL, Life Technologies, Auckland, New Zealand)/DMEM (Gibco, Invitrogen Corp., Auckland, New Zealand) for 2 d and then changed to 10% FBS/DMEM supplemented with 0.8% (wt/vol) in 5% FBS/DMEM and grown to 90% confluency. Cells were then seeded into 24-well plates in 5% FBS/DMEM for 24 h. Cells were grown arrested in 0.1% BSA (ICP, Auckland, New Zealand) for 24 h. Fresh media and experimental compounds were then added for a further 24 h. Cells were pulsed with [\(^{3}H\)]thymidine 4 h before the end of the experimental incubation. The experiment was terminated and cell counts or thymidine incorporation assessed. There were six wells in each group, and each experiment was repeated three or four times.

Cultures of primary human osteoblasts were prepared using normal human trabecular bone obtained from 50- to 70-yr-old patients undergoing knee or hip arthroplasty. Osteoblasts were grown from enzymetreated bone chips, using a modified method of Robey and Termine (16).

Cell lines were grown to 80% confluency in 5% fetal serum-supplemented media and then seeded into 24-well plates for proliferation assays, as described for primary rat osteoblasts. Human osteoblast-like cell line (SaOS-2) cells were seeded at a density of 7.0 x 10^5 cells/cm^2 and cultured in MEM. Murine bone marrow stromal cell line (ST2) cells were seeded at 1.4 x 10^5 cells/cm^2 in DMEM.

**Apoptosis assay**

For the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay, primary rat osteoblasts were seeded into 8-well chamber slides (Lab-Tek, Naperville, IL) in 5% fetal calf serum/DMEM (3 x 10^4 cells/chamber) and incubated for 24 h. Media were changed to 0.1% BSA/MEM and the cells incubated overnight. Fresh media and treatments were added and cells incubated a further 24 h. Cells were fixed in 2% paraformaldehyde for 15 min and washed thoroughly in PBS before being permeabilized with 1% Triton X-100 (BDH Chemicals Ltd., Poole, UK)/PBS for 5 min. Cells were rinsed thoroughly and then a modified TUNEL assay performed using the Dead End Colorimetric TUNEL system (Promega Corp., Madison, WI) according to manufacturer’s instructions. Results are expressed as the number of apoptotic bodies per microscopic field. There were eight wells for each group, and each experiment was repeated two to three times.

**Bone nodule assay**

Osteoblasts were isolated from 20-d fetal rat calvariae as described above. Cells were grown for 48 h in 10% FBS/DMEM in T75 flasks. Cells were then trypsinized (GIBCO, Invitrogen) and plated into 35-mm tissue culture dishes at a density of 3.5 x 10^3 cells/dish in 15% FBS/aMEM supplemented with 50 μg/ml l-ascorbic acid-2-phosphate (Sigma Chemical Co., St. Louis, MO). When cells were confluent (approximately 5 d), media were changed to 15% FBS/aMEM supplemented with l-asorbic acid-2-phosphate and 10 mM β-glycerophosphate (Sigma-Aldrich Co., St. Louis, MO), and test substances were added. These supplemented media were changed twice weekly and test substances were replaced. After 21 d the cells were fixed in neutral buffered formalin for 15 min, rinsed thoroughly with distilled water, and the cultures stained for mineral using Von Kossa stain. The number and area of mineralized bone nodules greater than 1 mm in diameter were quantified using image analysis.

**Chondrocyte culture**

Chondrocytes were isolated by removing cartilage (full-depth slices) from the tibial and femoral condyles of adult sheep under aseptic conditions. Slices were placed in 5% FBS/DMEM and chopped finely with a scalpel blade. Tissue was weighed and then incubated at 37°C in pronase solution (0.8% wt/vol in 5% FBS/DMEM) for 90 min followed by collagenase (0.1% wt/vol in 5% FBS/DMEM) for 18 h to complete the digestion. The cells were isolated from the digest by centrifugation (5 min at 1300 rpm), resuspended in 5% FBS/DMEM, passed through a nylon mesh screen of 90 μm pore size to remove any undigested fragments, and recentrifuged. The cells were washed twice and seeded into a 75-cm² flask containing 10% FBS/DMEM and ascorbic acid (50 μg/ml).

The cells were incubated under 5% CO2/95% air at 37°C. Confluence was reached within 7 d, at which time the cells were seeded into 24-well plates at a density of 1.4 x 10^5 cells/cm² in 5% FBS/DMEM + ascorbic acid. Proliferation assays were performed as for primary rat osteoblasts.

**Bone marrow culture**

Bone marrow was obtained from the long bones of normal Swiss male mice aged 4–6 wk, as previously described (15). Briefly, marrow cells were cultured for 2 h in 90-mm petri dishes. Nonadherent cells were then collected and grown in 48-well plates; 1.25 dihydroxyvitamin D3 (10⁻⁸ m) was added (d 0) to d 2 and 4, cultures were fed by removing 0.5 ml medium from each well and replacing with 0.5 ml fresh medium containing test substances and 1.25 dihydroxyvitamin D₃. After culture for 7 d, tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (containing three or more nuclei) were counted in all wells. Each experiment had three wells in which cells were grown on bone slices and checked for resorptive pits, indicating that the TRAP-positive multinucleated cells in these cultures were capable of resorbing bone. There were at least eight wells for each group, and each experiment was repeated three or four times.

**Mature isolated osteoclast culture**

Rat osteoclasts were isolated from the long bones of 1-d-old neonatal rats, as previously described (15). Briefly, an osteoclast-rich suspension prepared by homogenizing the bone tissue was placed onto bovine bone slices (9 mm²) in 96-well plates and incubated for 24 h to allow the mature osteoclasts to settle. The bone slices were then placed in 12-well plates (four slices per well) containing acidified media and incubated with test substances or vehicle for 24 h. After incubation, the bone slices were fixed and stained for TRAP. The number of TRAP-positive multinucleated cells (containing more than three nuclei) on each bone slice were counted, the cells were removed by gentle scrubbing, and then the bone slices were stained for 30 sec with toluidine blue. After several washes in water, the bone slices were dried and assessed for the pits excavated by the osteoclasts, using reflected light microscopic with metallurgical lenses. The results for each bone slice were expressed as the ratio of the number of pits to the number of osteoclasts. There were 6–12 bone slices in each group and each experiment was repeated two or three times.

**Real-time PCR**

RNA for real-time PCR was prepared from human primary osteoblasts and mouse bone marrow cultures. The culture conditions were as previously described for the proliferation assays and osteoclastogenesis assays, but for RNA preparation cells were plated at 6- well plates at densities of 1.5 x 10^5 primary osteoblasts/well and 4 x 10^5 bone marrow cells/well. Two wells were used for every experimental point. Bovine lactoferrin (100 μg/ml) or vehicle was added, and cells were harvested at the indicated time points after the beginning of treatment. Total cellular RNA was purified from the cultures using RNAse free DNase set (Qiagen, Valencia, CA), and genomic DNA was removed using RNase-free DNase set (Qiagen). Reverse transcription was carried out following the previously published protocol (17), and cDNA was used for real-time PCR. The primer-probe sets were purchased as Assay-on-Demand from Applied Biosystems (Foster City, CA). Multiplex PCR was performed with FAM-labeled specific probes [osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL) from human and OPG, RANKL, interferon-β and -γ, IL-18, and TNFα from mouse] and VIC-labeled 18S rRNA probes according to the company’s instructions, using ABI PRISM 7700 sequence detection system (Applied Biosystems). The experiments were performed in triplicates and repeated three times with similar results.

**Bone organ culture**

Mice were injected sc with 5 μCi ⁴⁵Ca at 2 d of age, and hemicalvariae were dissected out 4 d later. Hemicalvariae were preincubated for 24 h in medium 199 with 0.1% BSA and then changed to fresh medium containing test substances or vehicle. Incubation was continued for a further 48 h. To assess DNA synthesis, [³H]thymidine (0.6 μCi/ml) was added in the last 4 h of the incubation. The experiment was terminated.
and both calcium release and thymidine incorporation were assessed.
There were five to seven hemicalvariae in each group, and each
experiment was repeated three or four times.

Culture media for all studies described above contained penicillin
(100 U/ml) and streptomycin (100 μg/ml).

In vivo study

Five groups of sexually mature male mice were given daily sc injec-
tions over the right hemicalvaria for a consecutive 5 d, as previously
described (18, 19). Three groups (n = 15) received one of three doses
of bovine lactoferrin (0.04, 0.4, or 4 mg), and a further two groups (n = 9)
received vehicle or BSA, 4 mg. The animals were killed 10 d after the last
injection. Fluorochrome labels were injected sc at the base of the tail on
d 1 (calcein), 5 (alizarin red), and 14 (calcein). Calvariae were excised,
fixed in 10% neutral-buffered formalin, dehydrated, and embedded in
methylmethacrylate resin. Sections were cut, mounted on gelatin-coated
slides, and histomorphometric indices measured using image analysis.

All animal procedures were approved by the Animal Ethics Com-
mmittee of our institution.

Lactoferrin preparations

Bovine lactoferrin was isolated from fresh skim milk by cation ex-
change chromatography and gel filtration. Briefly, the milk at native pH
was passed through S Sepharose fast flow at 4 C and the bound proteins
eluted in steps with 0.1, 0.35, and 1 m NaCl, respectively. The 1 m NaCl
fraction containing lactoferrin was dialyzed and freeze dried. The re-
asulting material was then dissolved in 25 mM sodium phosphate buffer
(pH 6.5) and reapplied to the cation exchanger, which had been equili-
brated in the above buffer. Lactoferrin was eluted by application of a
salt gradient to 1 m NaCl in phosphate buffer and the recovered material
dialized and freeze-dried. Final purification of lactoferrin was achieved
by gel filtration through Sephacryl S300 in phosphate buffer and the
protein recovered as a dialyzed freeze-dried powder. Purity of the final
product was greater than 98% as assessed by resource reversed-phase
HPLC and mono-S HPLC (20).

Native human lactoferrin was prepared from breast milk as previ-
ously described (21) and fully saturated with iron by the addition of
ferric nitrilotriacetate. Full-length recombinant human lactoferrin was
expressed in baby hamster kidney cells, as described by Stowell et al. (22),
and was again used in its iron-saturated form. The degree of iron sat-
uration was determined from the spectral ratios A280/A466 and A412/
A466 (23), which have values of 20–22 and 0.70–0.74 for the fully iron-
saturated protein. The protein solutions were dialyzed exhaustively
against 0.05 m Tris-HCl (pH 8.0), 0.2 m NaCl, to remove any excess,
unbound, ferric iron before use.

Statistics

Data were analyzed using ANOVA with post hoc Dunnett’s tests. A
5% significance level is used throughout. Data are presented as means ±
se, unless indicated otherwise.

Results

Lactoferrin stimulates proliferation and differentiation of
osteoblast-like cells

Lactoferrin produced a dose-related increase in thymidine
incorporation in primary cultures of rat osteoblast-like cells
at 24 h (Fig. 1A). This effect was present at concentrations of
lactoferrin that occur in vivo (1–100 μg/ml). The same range
of concentrations of lactoferrin stimulated proliferation in
the human osteoblast-like cell line, SaOS-2 (Fig. 1B) (al-
though the size of the effect was less in these transformed
cells) and the stromal cell line, ST2 (Fig. 1C). Comparable
effects were found in primary cultures of human osteoblasts
based on cell proliferation (assessed
from thymidine incorporation) in pri-
mary cultures of rat osteoblast-like
cells (A); the human osteoblast-like cell
line, SaOS-2 (B); ST2, a stromal cell
line (C); and primary cultures of human
osteoblasts (D). Thymidine incorpo-
ration was measured during the last 4 h of
the 24-h incubation period. Data are
mean ± SEM. *, Significantly different
from control (P < 0.05).
liferation were complemented by an assessment of its action on differentiation of these cells. These studies were performed using 3-wk cultures of primary rat osteoblasts to assess bone nodule formation, a process that involves bone matrix deposition and mineralization, both of which are functions of differentiated osteoblasts. Lactoferrin, dose-dependently increased the number of nodules and the area of mineralized bone formed (Fig. 2). These effects were significant only at lactoferrin concentrations of 100 μg/ml and greater, suggesting that the concentrations needed to stimulate osteoblast differentiation are 10-fold greater than those required to stimulate proliferation in these models.

Lactoferrin prevents apoptosis in osteoblast-like cells

Lactoferrin decreased apoptosis observed in response to a 24-h period of serum deprivation in cultures of primary rat osteoblast-like cells, as judged by the number of TUNEL-positive cells (Fig. 3). This effect was dose dependent and evident at similar concentrations to those causing osteoblast proliferation. In this assay, lactoferrin reduced osteoblast apoptosis by up to 50–70%, indicating that lactoferrin not only stimulates osteoblast activity but also acts as a potent osteoblast survival factor.

Lactoferrin stimulates chondrocyte proliferation

In view of the mitogenic effects of lactoferrin on osteoblasts, its effect on proliferation of primary cultures of ovine chondrocytes was also assessed (Fig. 4). At concentrations of 10 and 20 μg/ml, lactoferrin increased thymidine incorporation in these cells. These effects were comparable in magnitude with those seen in osteoblasts.

Lactoferrin potently inhibits osteoclastogenesis but does not affect mature osteoclast activity

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 at concentrations of 10 μg/ml and greater (Fig. 5A). At 100 μg/ml, osteoclastogenesis was completely arrested. To determine at which stages of osteoclast development lactoferrin acts, it was added to bone marrow cultures from the outset or from d 2 (Fig. 5B). Both interventions reduced osteoclastogenesis, with more frequent additions, resulting in greater effects. This implies that lactoferrin acts on both preosteoclasts and more mature cells of this lineage. However, lactoferrin had no effect on bone resorption by isolated mature osteoclasts (Fig. 5C).

In light of this inhibition of osteoclastogenesis, the effects of lactoferrin on expression of RANKL and OPG were assessed in both osteoblasts and bone marrow cultures (Fig. 6). Exposure of osteoblasts to lactoferrin resulted in reduced OPG transcription and a transient increase in expression of RANKL. When these experiments were repeated in bone
marrow cultures, OPG mRNA levels decreased at 96 h but not at earlier time points. However, RANKL mRNA levels were diminished at both 72 and 96 h. Similar studies of expression of IL-18, TNF-α, interferon-β, and interferon-γ in bone marrow cultures showed no changes in response to treatment with lactoferrin (data not shown).

The effects of lactoferrin in neonatal mouse calvarial organ cultures were consistent with those from isolated cell cultures. Lactoferrin increased thymidine incorporation in calvariae (Fig. 7A), which previous studies in this model suggest probably reflects increased proliferation of cells of the osteoblast lineage (24), although effects on other cells in the calvariae might also have contributed. However, bone resorption, measured as 45Ca release, was unaffected (Fig. 7B). Because there is virtually no bone marrow in the calvariae, bone resorption in these explants predominantly reflects mature osteoclast function. Thus, this finding is consistent with that in isolated mature osteoclasts, shown in Fig. 5C.

**Lactoferrin increases bone growth in vivo**

The anabolic effect of lactoferrin on osteoblasts and its potent inhibition of osteoclastogenesis in vitro suggested that it might have positive effects on bone mass in vivo. To address this question, we administered lactoferrin, albumin, or vehicle over the right hemicalvaria of adult male mice for 5 consecutive days. Vehicle and albumin did not differ in their effects on any index so were pooled to provide a single control group. Sections from representative bones from control and lactoferrin-treated animals are shown in Fig. 8. The dramatic increases in bone area in the calvariae from lactoferrin-treated animals, compared with control, can be appreciated in these photomicrographs. New bone formation (assessed by measuring the distance between the first fluorochrome label and bone surface) was dose-dependently increased by lactoferrin, such that the 4-mg dose induced changes 4-fold greater than those observed in control animals (Fig. 9A). Local injections of lactoferrin also increased the mineral apposition rate (Fig. 9B) and the bone formation rate (Fig. 9C).

**Discussion**

This study provides the first evidence that lactoferrin is a promoter of osteoblast growth. In addition, it shows that lactoferrin is an inhibitor of osteoclastogenesis in vitro and increases local bone formation in vivo. Its effects on both the proliferation and survival of osteoblasts are profound, being far greater than those observed in response to several established osteoblast growth factors that we studied in the same in vitro assays. For instance, we found that maximal doses of epidermal growth factor, TGFβ, PTH, amylin, or insulin increase thymidine incorporation in the primary osteoblast...
cultures employed in this study by only 20–30% (25), whereas maximal doses of lactoferrin produce 3- to 5-fold increments. This growth-stimulating potency is complemented by the capacity of lactoferrin to substantially reduce osteoblast apoptosis. Again, this is much more dramatic than the effects that we have seen with other factors, such as IGF-1, which maximally decreases apoptosis in the TUNEL assay by 50% (26), compared with up to 70% with lactoferrin. In addition, lactoferrin promotes the function of differentiated osteoblasts, as seen in the bone nodule assay. Thus, lactoferrin acts to expand the pool of preosteoblastic cells by exerting mitogenic and antiapoptotic effects as well as driving differentiation of precursors to produce a more mature osteoblastic phenotype capable of promoting bone matrix deposition and mineralization. Each of these activities is likely to contribute to the potent effects on bone formation that we observed in vivo after administration of lactoferrin.

The actions of lactoferrin on osteoclasts are strikingly different from those we observed in osteoblasts, in that it produces an almost total arrest of osteoclastogenesis in mouse bone marrow cultures. Even though lactoferrin does not influence the activity of mature osteoclasts, the inhibition of osteoclastogenesis is still likely to result in a profound reduction in bone resorption. There is one previous report of the effects of lactoferrin on bone resorption, in which Lorget et al. (27) demonstrated that bovine lactoferrin reduces bone-resorbing activity in a rabbit mixed bone cell culture. This effect appeared to be mediated by an inhibition of the development of mature osteoclasts and operated by a mechanism independent of the RANK/RANKL/OPG system. The finding in the present study, that lactoferrin reduces RANKL expression in bone marrow cultures, could in part explain the inhibition of osteoclastogenesis, although this will tend to be counterbalanced by the effects of lactoferrin to also inhibit expression of OPG. We did not find changes in the levels of expression of other known regulators of osteoclastogenesis. It should be noted that lactoferrin has previously been demonstrated to inhibit the survival of progenitor cells in the bone marrow (28), implying that it might also act earlier in osteoclast development.

As a result of its effects on osteoblast growth, lactoferrin produces substantial increases in local bone formation in vivo, even with the very short-term exposure studied here. The bone growth resulting from local lactoferrin injection is considerably greater than we have found previously in response to factors such as insulin, amylin, adrenomedullin, C-terminal PTH-related peptide, calcitonin, or calcitonin gene-related peptide in the same model (29–32). It is qualitatively different from the effects of PTH in this model, which produces a powerful stimulation of bone resorption in addition to its effect on formation (33). The magnitude of the lactoferrin effect approaches those reported following local injection of statins (34) or TGFβ (35, 36). This potency is further attested to by 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 (see Fig. 8B) and the contralateral, uninjected, hemi-
calvariae (data not shown), something we have not seen with most other agents studied in this model. This anabolic po-
tency suggests that lactoferrin or its analogs should be ex-
plored as therapies for osteoporosis that can restore skeletal strength because most current interventions merely arrest further structural decline.

The present findings pose the question as to whether lac-
toferrin has a physiological role in skeletal development or homeostasis. Lactoferrin is expressed biphasically in embry-
ogenesis (7). It appears first in the two- to four-cell embryo. In the postblastocyst stage, its expression declines but in-
creases again dramatically in the latter half of gestation. Therefore, it could play a significant role in the development and function of chondrocytes and osteoblasts in the fetal skeleton. Lactoferrin is present in high concentrations in milk, particularly in colostrum, and it is likely that large proteins such as lactoferrin can cross the neonatal gut and enter the systemic circulation (37). Therefore, it is possible that the anabolic actions of lactoferrin might continue into the neonatal period. There is some evidence of biological effects of orally administered lactoferrin in adults (38–40), al-
though, as yet, none that it impacts on bone. Indeed, milk supplementation in human adults produces similar effects on bone density to calcium supplementation alone (41, 42), so it is unlikely that dietary lactoferrin has an important effect on human skeletal health later in life. Lactoferrin plays an important immunomodulatory function (14), decreasing the secretion of a number of osteolytic cytokines such as TNFα and IL-1β (11, 43, 44) and stabilizing mast cells (45). Thus, its
direct effects on the activity and development of bone cells may be complemented by these cytokine-mediated effects. In adult life, lactoferrin production is believed to be principally influenced by stimuli causing inflammation because it is
present in the secretory granules of neutrophils. Therefore, in inflammatory states, lactoferrin may play a role in coun-
terbalancing the catabolic effects on the skeleton of some of the mediators of the inflammatory response, although bone loss still predominates in most cases.

![Fig. 7. Effects of bovine lactoferrin (bLF) on thymidine incorporation (A) and bone resorption (B) (measured as release of 45Ca from pre-
labeled bones) in neonatal mouse calvariae. Thymidine incorporation, probably mainly reflecting osteoblast proliferation, was increased,
but there was no effect on bone resorption. Data are mean ± SEM. *Significantly different from control (P < 0.05).](image)

![Fig. 8. Photomicrographs of calvariae from animals treated with lactoferrin (4 mg) (A) and vehicle (B) for 5 d. Animals were killed 10 d later.
Fluorochrome labels used: green, calcein; red, alizarin. Two calcein labels were given 13 d apart. The increased new bone growth in the 13-d period (i.e. distance between the two green calcein labels, arrowed) can be appreciated in the calvaria from the lactoferrin-treated animal. These data are shown quantitatively in Fig. 9A. It can also be noted that there is new bone marrow formation occurring within the recently formed bone in the lactoferrin-treated calvaria. New bone formed on the injected side of the lactoferrin calvaria is partially woven (asterisk). This was seen only in the animals treated with the highest dose of lactoferrin and probably reflects the very high rate of matrix deposition. Horizontal bar, 50 μm.](image)
which are expressed in primary cultures of osteoblasts as well as in osteoblastic cell lines, have a large number of potential ligands (48), so they represent a novel pathway by which many factors might impact on bone cell function. Taken together, these data demonstrate that the naturally occurring glycoprotein, lactoferrin, is anabolic to bone in vivo, an effect that is consequent upon its potent proliferative, differentiating, and antiapoptotic actions in osteoblasts and its ability to inhibit osteoclastogenesis. Lactoferrin may therefore have a physiological role in bone growth. In addition, it is a potential therapeutic target in bone disorders such as osteoporosis and might have utility as a local agent to promote bone repair.

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Fig. 9. Effects of lactoferrin administered locally over the calvariae of adult male mice daily for 5 d in the doses indicated. Animals were killed 10 d later. The depth of new bone formed was assessed by measuring the distance between the first fluorochrome label and the bone surface. MAR, Mineral apposition rate; BFR/BS, bone formation rate expressed in relation to bone surface; bLF, bovine lactoferrin. Data are mean ± SEM. * Significantly different from control (P < 0.05).

Clearly, identification of the mechanism(s) by which lactoferrin acts on bone cells is important because of the potency of the effects demonstrated, and this is being actively investigated by our group (45a). A putative lactoferrin receptor in the intestine has been identified by Suzuki et al. (46), but we have not been able to detect this receptor in primary rat osteoblasts. However, we have presented preliminary evidence that lactoferrin acts through low-density lipoprotein receptor-related protein-1, a member of the low-density lipoprotein-related receptor family (47). These receptors,
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