

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

JILLIAN CORNISH, KAREN E. CALLON, DORIT NAOT, KATE P. PALMANO, TATJANA BANOVIC, USHA BAVA, MAUREEN WATSON, JIAN-MING LIN, P. C. TONG, QI CHEN, VINCENT A. CHAN, HELEN E. REID, NICK FAZZALARI, HEATHER M. BAKER, EDWARD N. BAKER, NEILL W. HAGGARTY, ANDREW B. GREY, AND IAN R. REID

Department of Medicine (J.C., K.E.C., D.N., T.B., U.B., M.W., J.-M.L., P.C.T., Q.C., V.A.C., H.E.R., A.B.G., I.R.R.) and School of Biological Sciences (H.M.B., E.N.B.), University of Auckland, Auckland 1001, New Zealand; Fonterra Research Centre (K.P.P., N.W.H.), Palmerston North 5315, New Zealand; and Institute of Medical and Veterinary Science (N.F.), Adelaide 5000, Australia

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 $\mu\text{g}/\text{ml}$). 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 mar-

row cultures, osteoclastogenesis was dose-dependently decreased and was completely arrested by lactoferrin, 100 $\mu\text{g}/\text{ml}$, 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 $\mu\text{g}/\text{ml}$ 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.

Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

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/MEM (Gibco, Invitrogen) and grown to 90% confluency. Cells were then seeded into 24-well plates in 5% FBS/MEM for 24 h. Cells were growth 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 [³H]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 enzyme-treated bone chips, using a modified method of Robey and Termine (16).

Cell lines were grown to 80% confluency in 5% fetal calf 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 7.0×10^3 cells/cm² and cultured in MEM. Murine bone marrow stromal cell line (ST2) cells were seeded at 1.4×10^4 cells/cm² 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, Nalge Nunc International, Naperville, IL) in 5% fetal calf serum/MEM (3×10^4 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 3.5×10^4 cells/dish in 15% FBS/ α MEM supplemented with 50 μ g/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/ α MEM supplemented with L-ascorbic 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 μ m 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 μ g/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 1.4×10^4 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 all wells except to negative controls. On 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 35 min 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 20 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 quantified, 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 microscopy 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 the cells were plated in 6-well plates at densities of 1.5×10^5 primary osteoblasts/well and 4×10^6 bone marrow cells/well. Two wells were used for every experimental point. Bovine lactoferrin (100 μ g/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 RNeasy minikit (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- κ B ligand (RANKL) from human and OPG, RANKL, interferon- β and - γ , IL-18, and TNF α 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 μ Ci 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 μ Ci/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 injections 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 Committee of our institution.

Lactoferrin preparations

Bovine lactoferrin was isolated from fresh skim milk by cation exchange 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 resulting material was then dissolved in 25 mM sodium phosphate buffer (pH 6.5) and reappplied 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 dialyzed 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 previously 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 saturation was determined from the spectral ratios A_{280}/A_{466} and A_{412}/A_{466} (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 \pm SE, unless indicated otherwise.

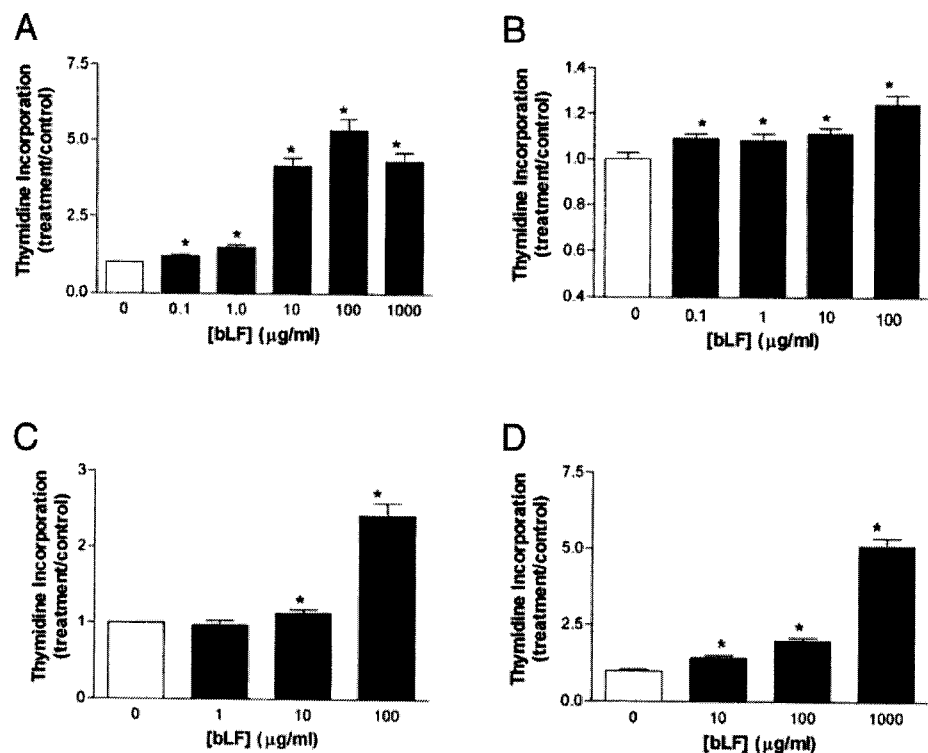
Results

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) (although 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 treated with bovine lactoferrin (Fig. 1D), and recombinant human lactoferrin was possibly more potent at low concentrations in these cells, increasing thymidine incorporation 2-fold at 10 µg/ml (data not shown). With this exception, the species of origin and method of preparation of the lactoferrin did not substantially impact the extent of the proliferative effect.

The above studies of lactoferrin action on osteoblast pro-

FIG. 1. Effects of bovine lactoferrin (bLF) on cell proliferation (assessed from thymidine incorporation) in primary 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 incorporation was measured during the last 4 h of the 24-h incubation period. Data are mean \pm 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 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$, 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 $\mu\text{g/ml}$ and greater (Fig. 5A). At 100 $\mu\text{g/ml}$, 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

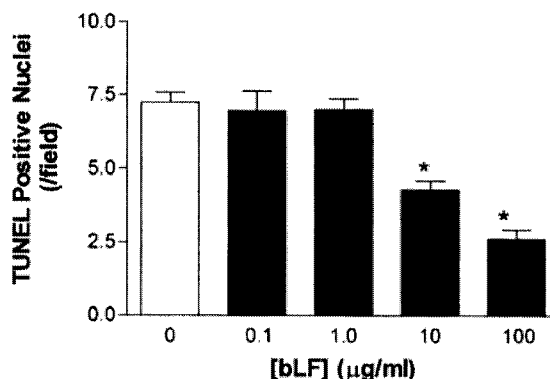
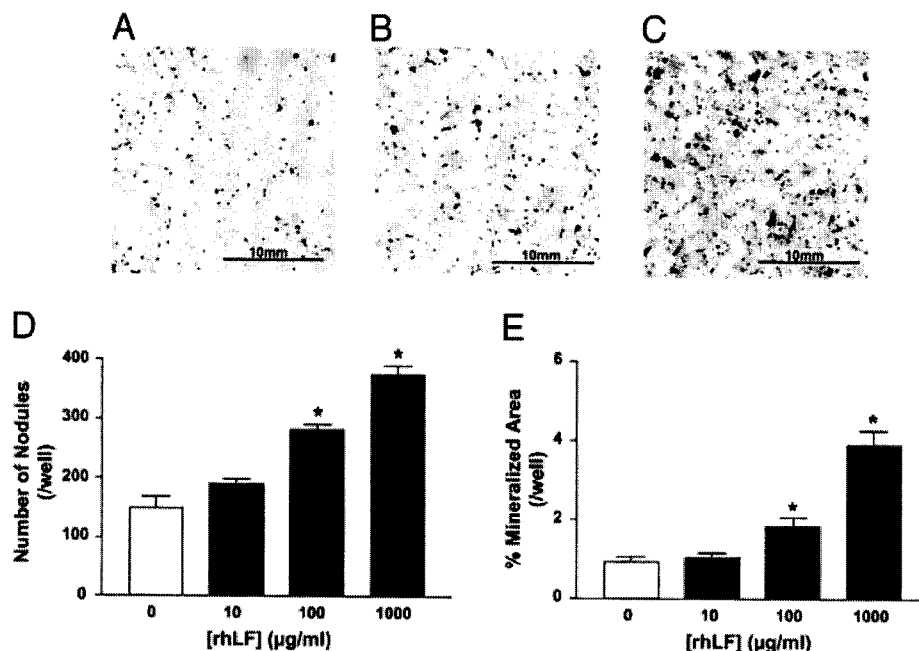


FIG. 3. Effect of bovine lactoferrin on apoptosis observed in response to a 24-h period of serum deprivation in cultures of primary rat osteoblast-like cells, as judged by the number of TUNEL-positive cells. Data are mean \pm SEM. *, Significantly different from control ($P < 0.05$).

FIG. 2. Effect of recombinant human lactoferrin (rhLF) on formation of bone nodules in primary cultures of rat osteoblast-like cells over a period of 3 wk. Cultures were stained for mineral using Von Kossa stain, and the number and area of mineralized bone nodules were quantified. A shows nodule formation in a control culture, whereas B and C were exposed to lactoferrin 100 and 1000 $\mu\text{g/ml}$, respectively. Quantification of nodule number and area are shown (D and E). Data are mean \pm SEM, *, Significantly different from control ($P < 0.05$).



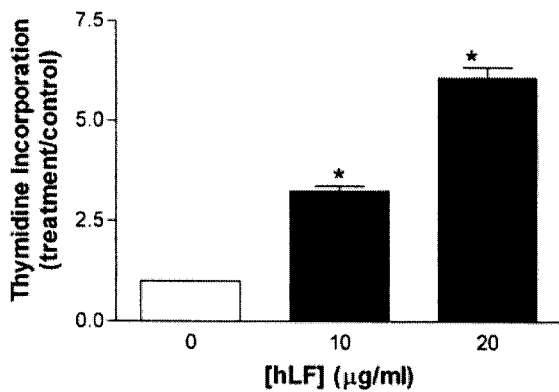


FIG. 4. Effect of human lactoferrin (hLF) on proliferation of primary cultures of ovine chondrocytes. Thymidine incorporation was measured during the last 4 h of the 24-h incubation period. Data are mean \pm SEM. *, Significantly different from control ($P < 0.05$).

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 ^{45}Ca 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).

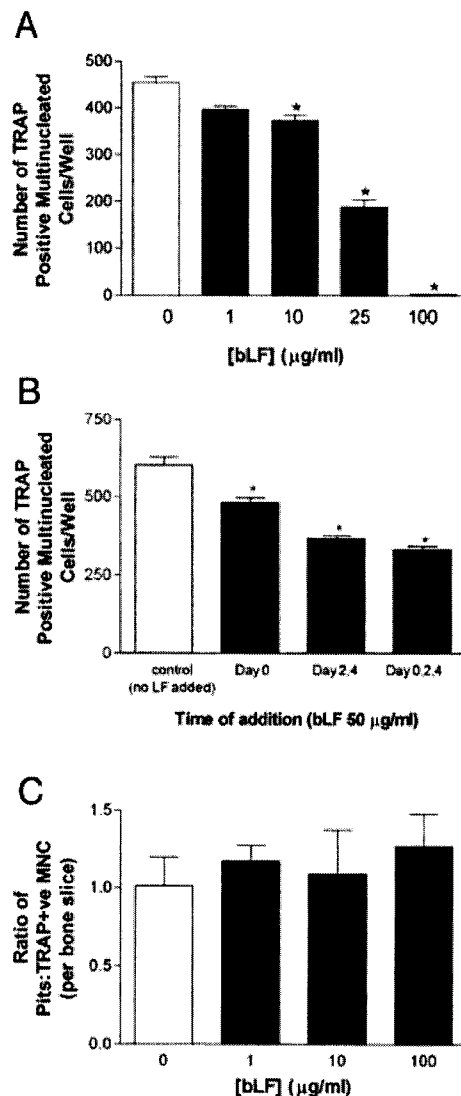


FIG. 5. A, Effects of bovine lactoferrin (bLF) on osteoclast development in mouse bone marrow cultures. The number of newly developed osteoclasts, assessed as multinucleated cells (MNCs) staining positively for TRAP, was significantly decreased by lactoferrin in concentrations of 10 $\mu\text{g/ml}$ and greater ($P < 0.05$). B, Effects of bovine lactoferrin, 50 $\mu\text{g/ml}$, on osteoclast development in mouse bone marrow cultures, according to time of addition (indicated on the x-axis). Each group is significantly different from all other groups. C, Effect of bovine lactoferrin on bone resorption by isolated mature osteoclasts (assessed as resorption pits per osteoclast). There were no statistically significant effects. Data are mean \pm SEM.

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, TGF β , PTH, amylin, or insulin increase thymidine incorporation in the primary osteoblast

Primary human osteoblast culture

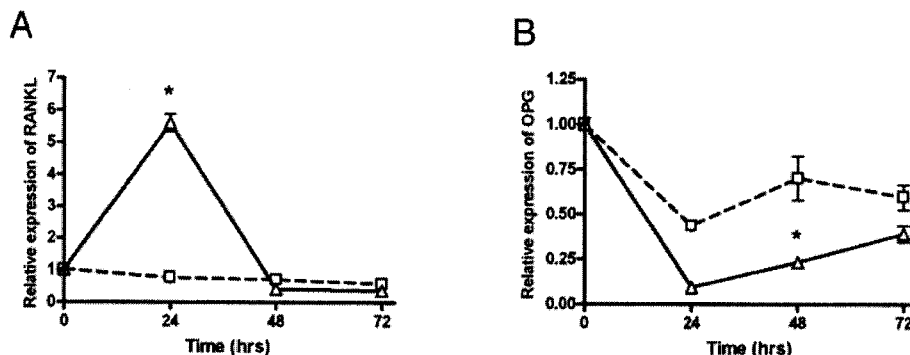
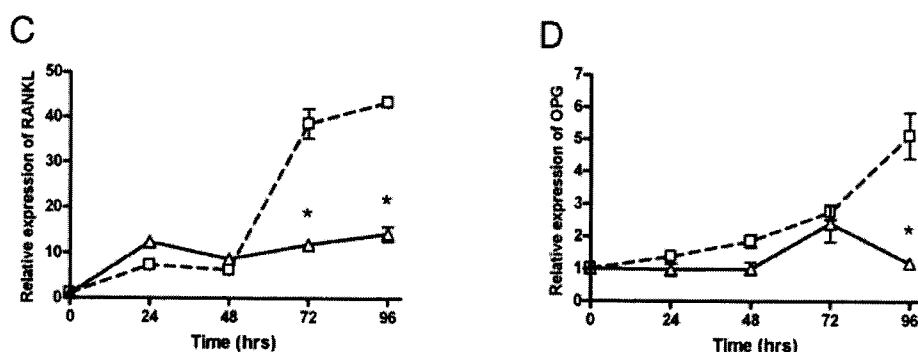


FIG. 6. Effect of bovine lactoferrin on RANKL and OPG expression in primary human osteoblast cultures (A and B) and mouse bone marrow cultures (C and D). Cells were incubated with lactoferrin at a concentration of 100 $\mu\text{g}/\text{ml}$ (Δ) or with vehicle (\square) for the duration of the experiment. The expression level of RANKL (A and C) and OPG (B and D) were determined in triplicates using multiplex real-time PCR. The relative expression levels were normalized to the level of 18S rRNA. Data are mean \pm SEM. *, Significantly different from control ($P < 0.05$). For some data points, error bars are not visible because they are smaller than the size of the symbol.

Mouse bone marrow culture



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 TGF β (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-

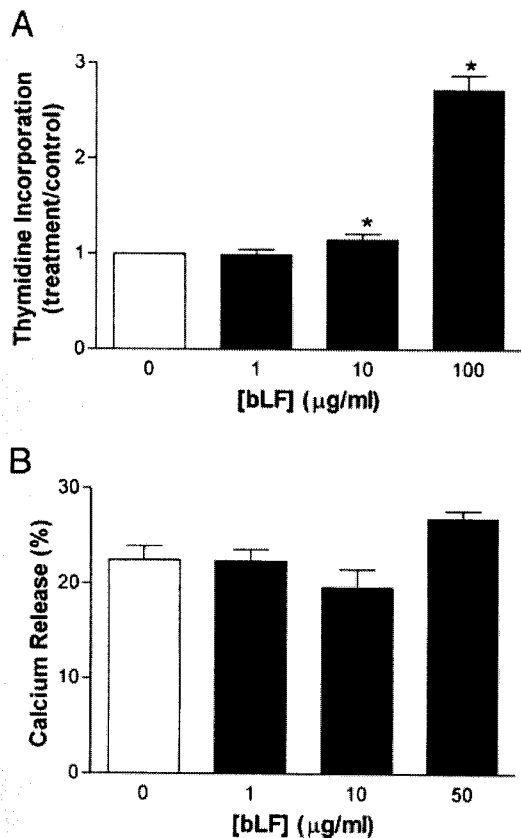


FIG. 7. Effects of bovine lactoferrin (bLF) on thymidine incorporation (A) and bone resorption (B) (measured as release of ^{45}Ca from pre-labeled bones) in neonatal mouse calvariae. Thymidine incorporation, probably mainly reflecting osteoblast proliferation, was increased, but there was no effect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

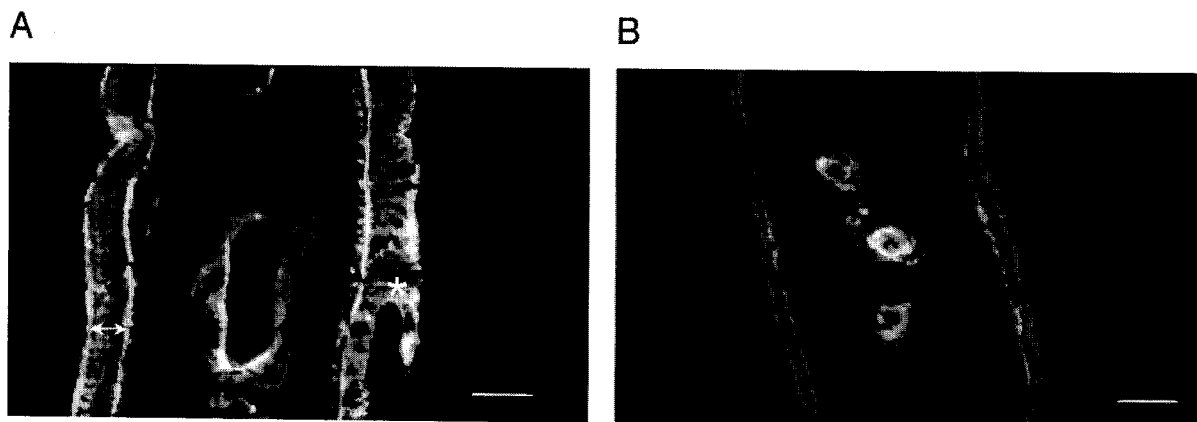


FIG. 8. Photomicrographs of calvariae from animals treated with lactoferrin (4 mg) (A) and vehicle (B) for 5 d. Animals were killed 10 d later. Fluorochrome labels used: green, calcein; red, alizarin. Two calcein labels were given 13 d apart. The increased new bone growth in the 13-d period (*i.e.* distance 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 .

calvariae (data not shown), something we have not seen with most other agents studied in this model. This anabolic potency suggests that lactoferrin or its analogs should be explored 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 lactoferrin has a physiological role in skeletal development or homeostasis. Lactoferrin is expressed biphasically in embryogenesis (7). It appears first in the two- to four-cell embryo. In the postblastocyst stage, its expression declines but increases 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), although, 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 $\text{TNF}\alpha$ and $\text{IL-1}\beta$ (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 counterbalancing the catabolic effects on the skeleton of some of the mediators of the inflammatory response, although bone loss still predominates in most cases.

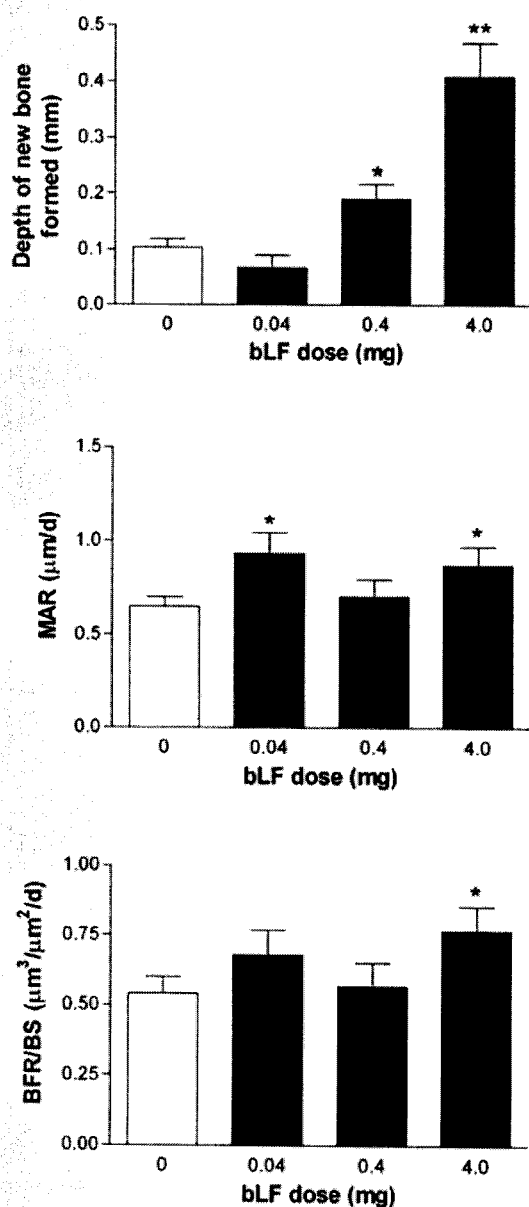


FIG. 9. Effects of lactoferrin administered locally over the calvariae of adult male mice daily for 5 d in the doses indicated. Animals were killed 10 d later. The depth of new bone formed was assessed by measuring the distance between the first fluorochrome label and the bone surface. MAR, Mineral apposition rate; BFR/BS, bone formation rate expressed in relation to bone surface; bLF, bovine lactoferrin. Data are mean \pm 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,

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.

Acknowledgments

Doreen Presnall's assistance with the preparation of the manuscript is gratefully acknowledged.

Received September 30, 2003. Accepted May 18, 2004.

Address all correspondence and requests for reprints to: Dr. J. Cornish, Department of Medicine, University of Auckland, Private Bag 92019, Auckland 1001, New Zealand. E-mail: j.cornish@auckland.ac.nz.

This work was supported by New Zealand Milk, the New Zealand Dairy Board, the Lactopharma Consortium, and the Health Research Council of New Zealand.

References

- Metz-Boutigue MH, Jolles J, Mazurier J, Schoentgen F, Legrand D, Spik G, Montreuil J, Jolles P 1984 Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins. *Eur J Biochem* 145:659–676
- Lonnerdal B, Iyer S 1995 Lactoferrin: molecular structure and biological function. *Annu Rev Nutr* 15:93–110
- Dinauer MC, Lekstrom-Himes JA, Dale DC 2000 Inherited neutrophil disorders: molecular basis and new therapies. *Hematology (Am Soc Hematol Educ Program)* 1:303–318
- Caccavo D, Sebastiani GD, Di Monaco C, Guido F, Galeazzi M, Ferri GM, Bonomo L, Afeltra A 1999 Increased levels of lactoferrin in synovial fluid but not in serum from patients with rheumatoid arthritis. *Int J Clin Lab Res* 29:30–35
- Weinberg ED 2001 Human lactoferrin: a novel therapeutic with broad spectrum potential. *J Pharm Pharmacol* 53:1303–1310
- Bi BY, Lefebvre AM, Dus D, Spik G, Mazurier J 1997 Effect of lactoferrin on proliferation and differentiation of the Jurkat human lymphoblastic T cell line. *Arch Immunol Ther Exp (Warsz)* 45:315–320
- Ward PP, Mendoza-Meneses M, Mulac-Jericevic B, Cunningham GA, Saucedo-Cardenas O, Teng CT, Conneely OM 1999 Restricted spatiotemporal expression of lactoferrin during murine embryonic development. *Endocrinology* 140:1852–1860
- Sawatzki G, Rich IN 1989 Lactoferrin stimulates colony stimulating factor production *in vitro* and *in vivo*. *Blood Cells* 15:371–385
- Baveye S, Ellass E, Fernig DG, Blanquart C, Mazurier J, Legrand D 2000 Human lactoferrin interacts with soluble CD14 and inhibits expression of endothelial adhesion molecules, E-selectin and ICAM-1, induced by the CD14-lipopopolysaccharide complex. *Infect Immun* 68:6519–6525
- Guillen C, McInnes IB, Vaughan DM, Kommajosyula S, Van Berkel PH, Leung BP, Aguila A, Brock JH 2002 Enhanced Th1 response to *Staphylococcus aureus* infection in human lactoferrin-transgenic mice. *J Immunol* 168:3950–3957
- Kimber I, Cumberbatch M, Dearman RJ, Headon DR, Bhushan M, Griffiths CE 2002 Lactoferrin: influences on Langerhans cells, epidermal cytokines, and cutaneous inflammation. *Biochem Cell Biol* 80:103–107
- Elass E, Masson M, Mazurier J, Legrand D 2002 Lactoferrin inhibits the lipopolysaccharide-induced expression and proteoglycan-binding ability of interleukin-8 in human endothelial cells. *Infect Immun* 70:1860–1866
- Frydecka I, Zimecki M, Bocko D, Kosmaczewska A, Teodorowska R, Ciszak L, Kruzel M, Wlodarska-Polinska J, Kuliczowski K, Kornafel J 2002 Lactoferrin-induced up-regulation of zeta (ζ) chain expression in peripheral blood T lymphocytes from cervical cancer patients. *Anticancer Res* 22:1897–1901
- Baveye S, Ellass E, Mazurier J, Spik G, Legrand D 1999 Lactoferrin: a multifunctional glycoprotein involved in the modulation of the inflammatory process. *Clin Chem Lab Med* 37:281–286
- Cornish J, Callon KE, Lin CQ, Xiao CL, Mulvey TB, Cooper GJ, Reid IR 1999

- Trifluoroacetate, a contaminant in purified proteins, inhibits proliferation of osteoblasts and chondrocytes. *Am J Physiol* 277:E779–E783
16. Robey PG, Termine JD 1985 Human bone cells *in vitro*. *Calcif Tissue Int* 37:453–460
 17. Naot D, Callon KE, Grey A, Cooper GJS, Reid IR, Cornish J 2001 A potential role for adrenomedullin as a local regulator of bone growth. *Endocrinology* 142:1849–1857
 18. Cornish J, Callon KE, Gasser JA, Bava U, Gardiner EM, Coy DH, Cooper GJS, Reid IR 2000 Systemic administration of a novel octapeptide, amylin-(1–8), increases bone volume in male mice. *Am J Physiol* 279:E730–E735
 19. Cornish J, Callon K, King A, Edgar S, Reid IR 1993 The effect of leukemia inhibitory factor on bone *in vivo*. *Endocrinology* 132:1359–1366
 20. Palmano KP, Elgar DF 2002 Detection and quantitation of lactoferrin in bovine whey samples by reversed-phase high-performance liquid chromatography on polystyrene-divinylbenzene. *J Chromatogr A* 947:307–311
 21. Baker EN, Rumball SV 1977 Crystallographic data for human lactoferrin. *J Mol Biol* 111:207–210
 22. Stowell KM, Rado TA, Funk WD, Tweedie JW 1991 Expression of cloned human lactoferrin in baby-hamster kidney cells. *Biochem J* 276:349–355
 23. Aisen P, Leibman A 1972 Lactoferrin and transferrin: a comparative study. *Biochim Biophys Acta* 257:314–323
 24. Cornish J, Callon KE, Edgar SG, Reid IR 1997 Leukemia inhibitory factor is mitogenic to osteoblasts. *Bone* 21:243–247
 25. Cornish J, Callon KE, Lin CQ, Xiao CL, Gamble GD, Cooper GJ, Reid IR 1999 Comparison of the effects of calcitonin gene-related peptide and amylin on osteoblasts. *J Bone Miner Res* 14:1302–1309
 26. Cornish J, Balchin LM, Callon KE, Grey AB, Cooper GJS, Reid IR 2000 Amylin and adrenomedullin—novel promoters of osteoblast survival from apoptosis. *Bone* 27:195
 27. Lorget F, Clough J, Oliveira M, Daury MC, Sabokbar A, Offord E 2002 Lactoferrin reduces *in vitro* osteoclast differentiation and resorbing activity. *Biochem Biophys Res Commun* 296:261–266
 28. Hangoc G, Falkenburg JH, Broxmeyer HE 1991 Influence of T-lymphocytes and lactoferrin on the survival-promoting effects of IL-1 and IL-6 on human bone marrow granulocyte-macrophage and erythroid progenitor cells. *Exp Hematol* 19:697–703
 29. Cornish J, Callon KE, Reid IR 1996 Insulin increases histomorphometric indices of bone formation *in vivo*. *Calcif Tissue Int* 59:492–495
 30. Cornish J, Callon KE, Nicholson GC, Reid IR 1997 Parathyroid hormone-related protein-(107–139) inhibits bone resorption *in vivo*. *Endocrinology* 138:1299–1304
 31. Cornish J, Callon KE, Coy DH, Jiang NY, Xiao L, Cooper GJ, Reid IR 1997 Adrenomedullin is a potent stimulator of osteoblastic activity *in vitro* and *in vivo*. *Am J Physiol* 273:E1113–E1120
 32. Cornish J, Callon KE, Cooper GJ, Reid IR 1995 Amylin stimulates osteoblast proliferation and increases mineralized bone volume in adult mice. *Biochem Biophys Res Commun* 207:133–139
 33. Cornish J, Callon KE, Reid IR 1995 An *in vivo* model for the rapid assessment of the local effects of parathyroid hormone on bone histomorphometry. *Bone* 17(Suppl 4):S249–S254
 34. Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, Boyce B, Zhao M, Gutierrez G 1999 Stimulation of bone formation *in vitro* and in rodents by statins. *Science* 286:1946–1949
 35. Marcelli C, Yates AJ, Mundy GR 1990 *In vivo* effects of human recombinant transforming growth factor β on bone turnover in normal mice. *J Bone Miner Res* 5:1087–1096
 36. Mackie EJ, Trechsel U 1990 Stimulation of bone formation *in vivo* by transforming growth factor- β : remodeling of woven bone and lack of inhibition by indomethacin. *Bone* 11:295–300
 37. Hutchens TW, Henry JF, Yip TT 1991 Structurally intact (78-kDa) forms of maternal lactoferrin purified from urine of preterm infants fed human milk: identification of a trypsin-like proteolytic cleavage event *in vivo* that does not result in fragment dissociation. *Proc Natl Acad Sci USA* 88:2994–2998
 38. Zimecki M, Dawiskiba J, Zawirska B, Krawczyk Z, Kruzel M 2003 Bovine lactoferrin decreases histopathological changes in the liver and regulates cytokine production by splenocytes of obstructive jaundiced rats. *Inflamm Res* 52:305–310
 39. Wakabayashi H, Takakura N, Teraguchi S, Tamura Y 2003 Lactoferrin feeding augments peritoneal macrophage activities in mice intraperitoneally injected with inactivated *Candida albicans*. *Microbiol Immunol* 47:37–43
 40. Kumari J, Swain T, Sahoo PK 2003 Dietary bovine lactoferrin induces changes in immunity level and disease resistance in Asian catfish *Clarias batrachus*. *Vet Immunol Immunopathol* 94:1–9
 41. Lau EMC, Woo J, Lam V, Hong A 2001 Milk supplementation of the diet of postmenopausal Chinese women on a low calcium intake retards bone loss. *J Bone Miner Res* 16:1704–1709
 42. Reid IR, Ames RW, Evans MC, Gamble GD, Sharpe SJ 1993 Effect of calcium supplementation on bone loss in postmenopausal women. *N Engl J Med* 328:460–464
 43. Zimecki M, Wlasczyk A, Zagulski T, Kubler A 1998 Lactoferrin lowers serum interleukin 6 and tumor necrosis factor α levels in mice subjected to surgery. *Arch Immunol Ther Exp (Warsz)* 46:97–104
 44. Zimecki M, Miedzybrodzki R, Szymaniec S 1998 Oral treatment of rats with bovine lactoferrin inhibits carrageenan-induced inflammation; correlation with decreased cytokine production. *Arch Immunol Ther Exp (Warsz)* 46:361–365
 45. He S, McEuen AR, Blewett SA, Li P, Buckley MG, Leufkens P, Walls AF 2003 The inhibition of mast cell activation by neutrophil lactoferrin: uptake by mast cells and interaction with trypsinase, chymase and cathepsin G. *Biochem Pharmacol* 65:1007–1015
 - 45a. Grey A, Banovic T, Zhu Q, Watson M, Callon K, Palmano K, Ross J, Naot D, Reid IR, Cornish J, The low density lipoprotein-receptor-related protein 1 (LRP1) is a mitogenic receptor for lactoferrin in osteoblastic cells. *Mol Endocrinol*, in press
 46. Suzuki YA, Shin K, Lonnerdal B 2001 Molecular cloning and functional expression of a human intestinal lactoferrin receptor. *Biochemistry* 40:15771–15779
 47. Grey A, Banovic T, Callon K, Palmano K, Lin JM, Chan V, Bava U, Reid IR, Cornish J 2003 Lactoferrin, a potent anabolic factor in bone, signals through the low-density lipoprotein receptor-related protein (LRP) 1. *Bone* 32:S88–S89
 48. Strickland DK, Gonias SL, Argraves WS 2002 Diverse roles for the LDL receptor family. *Trends Endocrinol Metab* 13:66–74

Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.