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Lactoferrin as an effector molecule in the skeleton

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Abstract Lactoferrin is a pleiotropic factor with potent antimicrobial and immunomodulatory activities. In recent years, studies have shown that lactoferrin also acts on the skeleton to promote bone growth. Lactoferrin stimulates the proliferation and differentiation of the bone forming cells, the osteoblasts, and acts as a survival factor for these cells. Lactoferrin also inhibits osteoclastogenesis, reducing the number of cells that can actively resorb bone, thus producing a greater overall increase in bone volume. In vivo, local injection of lactoferrin results in substantial increases in bone area, establishing lactoferrin as an effector molecule in the skeleton. Investigations of the mechanism of action of lactoferrin in bone cells showed that the mitogenic effect of lactoferrin in osteoblasts is mediated mainly through LRP1, a member of the low density lipoprotein receptor-related proteins. Lactoferrin induces activation of p42/44 MAPK signaling as well as PI3-kinase-dependent phosphorylation of Akt in osteoblasts. Differential gene expression studies indicated a possible role for the activation of IGF1, Ptgs2 and Nfatc1 in mediating the mitogenic activity of lactoferrin in osteoblasts. Lactoferrin is a positive regulator of bone with a possible physiological role in bone growth and healing. There is a growing interest in the potential use of lactoferrin for the improvement of bone health, and in a number of recent studies dietary lactoferrin supplementation improved bone mineral density and bone strength. Lactoferrin appears to be a promising candidate for the development of an anabolic therapeutic factor for osteoporosis.

Keywords Lactoferrin · Bone · Osteoblast · Osteoclast · Anabolic

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Introduction

A healthy skeleton is maintained by being continually replaced. This remodeling process occurs throughout life, completing a full replacement of the skeleton every 8–10 years. Bone remodeling depends on the action of two main bone cells, the osteoclasts which resorb old bone and the osteoblasts that form new bone by firstly laying down an unmineralized matrix and then orchestrating its mineralization. The precursors of both the populations of cells reside in the bone marrow, and the osteoblasts and osteoclasts are in close communication and regulate each other's function. Good bone health depends on the regulation of bone remodeling by numerous local and circulating factors. Many bone diseases are characterized by an imbalance between the activities of osteoclasts and osteoblasts, with osteoporosis being the most common bone disease and a major cause of morbidity and

health expenditure in aging populations. Antiresorptive medications such as bisphosphonates and estrogen represent the main pharmacological treatment options for patients with osteoporosis. Despite their great therapeutic value, these agents are limited in their ability to restore bone mass and patients would greatly benefit from the development of new anabolic therapies designed to increase bone mineral density.

There is a growing interest in the potential use of lactoferrin for the improvement of bone health. Lactoferrin is an 80 kDa iron binding glycoprotein that belongs to the transferrin family and it is produced by epithelial cells of exocrine glands. Lactoferrin is present in very high concentrations in colostrum and milk. In healthy subjects, lactoferrin circulates at 2–7 μg/ml and is predominantly neutrophil-derived so that during inflammation lactoferrin can reach much higher local concentrations. In bone, lactoferrin functions as a growth factor, acting at physiological concentrations to induce osteoblast growth and activity and inhibit osteoclast development in vitro and to promote bone growth in vivo. In this review, we discuss studies that have established lactoferrin as an anabolic factor in bone; we present investigations into the mechanisms of action of lactoferrin in bone cells and recent studies showing a possible use of lactoferrin as a dietary supplement for the improvement of bone health.

Lactoferrin's effects on osteoblasts

Investigations by our group and others established that lactoferrin acts as a growth factor in bone tissue at physiological concentrations. Lactoferrin potently induces proliferation of primary osteoblasts and osteoblastic-cell lines and increases osteoblast differentiation (Cornish et al. 2004; Takayama and Mizumachi 2008, 2009). In studies performed using 3-week cultures of primary rat osteoblasts to assess bone nodule formation, a process that involves bone matrix deposition and mineralization by differentiated osteoblasts, lactoferrin dose-dependently increased the number of nodules and the area of mineralized bone formed (Cornish et al. 2004). Investigations have also demonstrated the potential use of lactoferrin as an osteogenic growth factor in bone tissue engineering, showing that cells of the human osteoblastic line MG63 plated on lactoferrin-embedded collagen membranes had increased alkaline phosphatase activity and osteocalcin production (Takayama and Mizumachi 2009). The degree of iron-loading has little effect on the mitogenic activity of lactoferrin in osteoblasts. Similar stimulation of osteoblast proliferation could be induced by iron-depleted (apo) and iron-loaded (holo) bovine lactoferrin and the proliferative effect was also retained when the iron was substituted with either chromium or manganese cations (Cornish et al. 2006).

Of the various lactoferrin receptors that have been described (Ji and Mahley 1994; Legrand et al. 2004; Suzuki et al. 2001; Willnow et al. 1992) we found that the low density lipoprotein receptor-related protein 1 and 2 (LRP1 and LRP2), which are multiligand members of the LRP family of endocytic receptors, are present on osteoblastic cells (Grey et al. 2004). LRP1 is at least partially responsible for lactoferrin's mitogenic effects in osteoblasts (Grey et al. 2004). The 39 kDa receptor-associated protein (RAP), is an intracellular chaperone in the biosynthesis of LRP1 and 2, preventing premature ligand binding during receptor trafficking but it can be also be used as a useful tool in vitro as it can be added externally to the cells and specifically inhibits ligand binding to both receptors (Vash et al. 1998). Thus utilizing RAP we were able to demonstrate that lactoferrin is endocytosed into cytoplasmic membrane-bound vacuoles by osteoblastic cells via a mechanism that involves functional LRP1 (Grey et al. 2004). In addition to endocytosis, LRP1 functions as a signaling receptor in osteoblasts, activating p42/44 MAP kinases and unlike endocytosis which is not required for the mitogenic effects of lactoferrin, activation of the MAP kinase pathway is essential for mitogenesis.

The effect of lactoferrin on apoptosis of osteoblasts was also studied, as the inhibition of cell death is an additional way to expand the population of osteoblasts. Lactoferrin potently protects osteoblastic cells from apoptosis induced by serum withdrawal (Grey et al. 2006). Surprisingly, this effect was not sensitive to the LRP1 inhibitor, RAP. Neither did lactoferrin selectively prevent apoptosis in fibroblastic cells expressing wild-type LRP1 compared to LRP1-null fibroblasts. Lactoferrin activates PI3 kinase-dependent Akt signaling but this effect is neither LRP1-dependent nor required for lactoferrin-induced cell survival. Inhibiting the activation of p42/44 MAP kinases pathway

does not abrogate lactoferrin's pro-survival actions either. These results suggest that the molecular mechanisms that underpin the ability of lactoferrin to promote cell survival differ fundamentally from those which subserve its mitogenic actions, in particular being mediated by a distinct cell-membrane-based receptor.

Further investigations of the mechanism of action of lactoferrin in osteoblasts are currently underway. We have used microarrays and low-density arrays to study changes in gene expression induced by lactoferrin treatment in osteoblasts (Naot et al. manuscript submitted). In human primary osteoblasts we identified a significant up-regulation of IGF1 mRNA and down-regulation of dickkopf homolog 1 (DKK1). In osteoblasts from rodents we determined increases in transcripts of a number of genes, including prostaglandin-endoperoxide synthase 2 (Ptgs2) and nuclear factor of activated T cells-1 (Nfatc1). The significance of these changes to the mitogenic effect of lactoferrin in osteoblasts and its ability to promote osteoblast differentiation are currently being investigated.

Although the positive effects of lactoferrin on osteoblast proliferation and differentiation have been well established the mechanisms involved in these activities are still unclear. A number of pathways activated by lactoferrin in osteoblasts have been identified but the sequence of events that follows the exposure of the osteoblast to lactoferrin and the details of the crosstalk between the downstream pathways remain to be determined (Fig. 1).

Lactoferrin's effects on osteoclasts

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 (Cornish et al. 2004). The number of newly developed osteoclasts, assessed as multinucleated cells staining positively for tartrate-resistant acid phosphatase (TRAP), was significantly decreased by lactoferrin

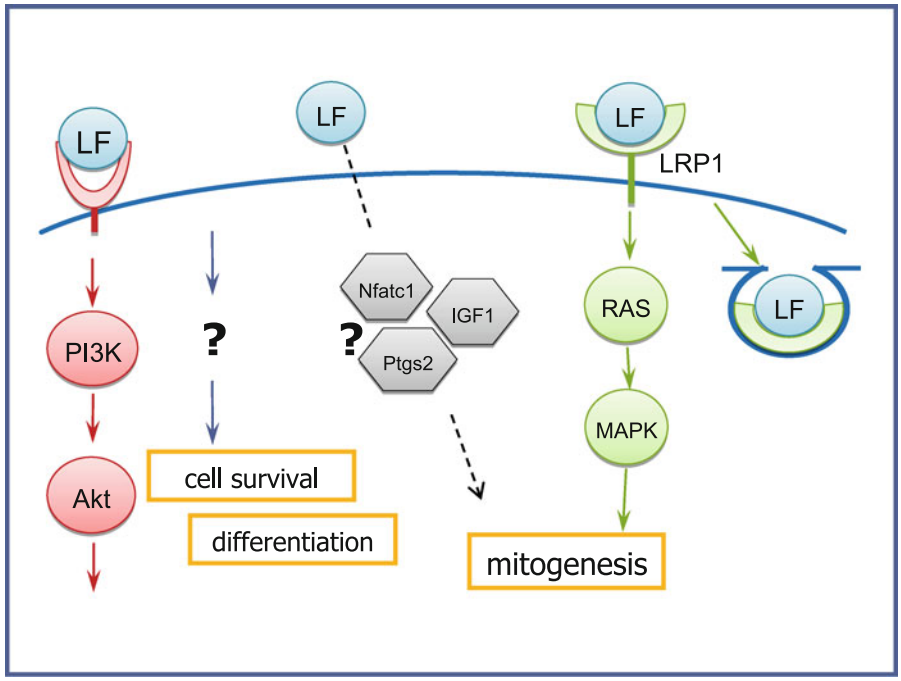

Fig. 1 Mechanisms of action of lactoferrin in osteoblasts. Binding of lactoferrin to LRP1 receptor on osteoblasts induces endocytosis of the lactoferrin-LRP1 complex as well as activation of the p42/44 MAP kinases signaling pathway. Inhibition of the MAPK pathway blocks the mitogenic effect of lactoferrin in osteoblasts. Lactoferrin also activates the PI3K/Akt signaling pathway and increases mRNA levels of IGF1, Nfatc1 and Ptgs2 but the functional significance of these remains to be determined. The mechanisms involved in the activities of lactoferrin as a survival factor and as a promoter of osteoblast differentiation are unknown yet

at concentrations of 10 μg/ml, and at 100 μg/ml osteoclastogenesis was completely arrested. In another study of the effects of lactoferrin on bone resorption, bovine lactoferrin was found to reduce bone-resorbing activity in a rabbit mixed bone cell culture (Lorget et al. 2002). In contrast to its inhibitory effect on osteoclast development, lactoferrin had no effect on bone resorption by isolated mature osteoclasts nor in organ cultures which also detect mature osteoclast activity (Cornish et al. 2004). Taken together, these results indicate that although lactoferrin does not affect the activity of fully differentiated osteoclasts to resorb bone, it inhibits bone resorption by reducing the number of osteoclasts formed from precursor cells.

Factors that regulate osteoclast differentiation and activity use direct mechanisms, indirect mechanisms or a combination of both. Some factors can directly affect osteoclastic cells and their precursors whereas others act on osteoblasts to modify the expression of proteins which in turn control osteoclastogenesis. The bone marrow derived culture systems used for osteoclastogenesis studies cannot be used to distinguish between direct and indirect effects as both osteoblast and osteoclast precursors are present in the cultures. Cells of the mouse macrophage line RAW264.7 can be induced to differentiate in vitro to osteoclasts by the addition of receptor activator for nuclear factor κB ligand (RANKL) to the culture medium. This experimental system is used to determine the ability of factors to act directly on osteoclasts. We have recently shown that lactoferrin inhibited RANKL-induced osteoclastogenesis in RAW264.7 cells in a dose-dependent manner, implying a direct effect on osteoclast-like cells, independent of the presence of osteoblasts (Fig. 2a). The mechanisms involved in lactoferrin's action on osteoclasts are unknown, and the receptor which mediates lactoferrin's inhibitory effect on osteoclasts hasn't been identified yet. The addition of the LRP1 inhibitor RAP did not alter lactoferrin-induced inhibition of osteoclastogenesis (Fig. 2b) indicating that the receptor involved in this inhibition is not LRP1.

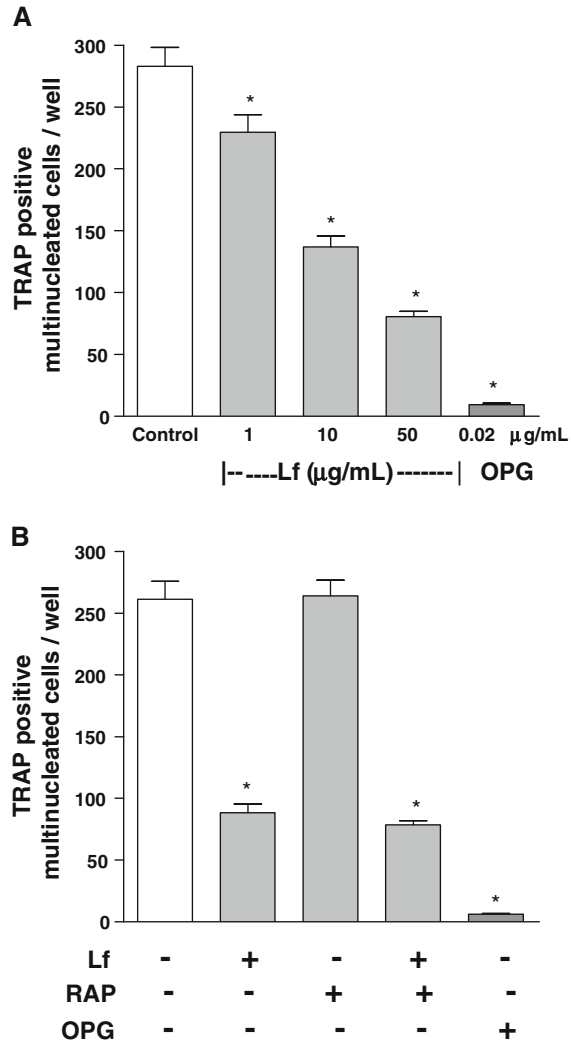

Fig. 2 Lactoferrin directly inhibits osteoclastogenesis in RAW264.7 cells independently of LRP signaling. **a** RAW264.7 cells, induced to differentiate to osteoclasts by the addition of 50 ng/ml RANKL to the culture media, were treated with increasing concentrations of lactoferrin. TRAP positive multinucleated cells were counted on day 5. OPG (osteoprotegerin), a very potent inhibitor of RANKL-induced osteoclastogenesis was used as a positive control. **b** The activity of lactoferrin to inhibit osteoclastogenesis was not affected by the addition of RAP. In this experiment lactoferrin was used at a concentration of 50 μg/ml, RAP at 10 nM and OPG at 20 ng/ml. Data are presented as the mean + SEM. * $P < 0.05$. *Lf* lactoferrin, *OPG* osteoprotegerin, *RAP* receptor-associated protein, *TRAP* tartrate-resistant acid phosphatase

Lactoferrin increases bone growth in vivo

A local injection model was used to study the effect of lactoferrin on bone in vivo (Cornish et al. 2004). Lactoferrin was administered over the skulls of adult male mice for 5 consecutive days, resulting in a greatly increased new bone formation in the lactoferrin-injected animals compared to controls. Assessment

of dynamic histomorphometric indices demonstrated significant increases in mineral apposition rate and bone formation rate induced by lactoferrin. The potent effect of lactoferrin on bone area in vivo could be the result of the combined anabolic effects of lactoferrin on osteoblasts and the inhibitory effect on osteoclastogenesis that were seen in vitro.

A number of recently published studies tested the potential use of lactoferrin for protection against bone loss. Two of the studies used ovariectomized (OVX) rodents as a model for post menopausal bone loss and measured the effect of dietary supplementation of lactoferrin on bone (Blais et al. 2009; Guo et al. 2009). Immunoreactive bovine lactoferrin was detected in the peripheral blood of OVX mice fed with a lactoferrin-supplemented diet, and following 27 weeks of treatment these mice showed higher bone mineral density and bone strength compared to a control group (Blais et al. 2009). In a second study, lactoferrin orally administered to OVX rats for 3 months protected them against the OVX-induced reduction of bone volume and bone mineral density and increased the parameters of mechanical strength. Measurements of biochemical markers of bone remodeling indicated greater bone formation and reduced bone resorption occurred in rats treated with lactoferrin (Guo et al. 2009). In a clinical study of 38 healthy postmenopausal women who were randomized to receive placebo or RNAse-enriched-lactoferrin by mouth, there was a significant reduction in bone resorption markers and an increase in osteoblast markers in the lactoferrin-treated group (Bharadwaj et al. 2009).

Conclusions

Lactoferrin has a positive effect on bone tissue and might be useful in pathological states of reduced bone density when used either systemically or locally. The molecular mechanisms activated by lactoferrin in osteoclasts are unknown and those activated in osteoblasts are only partially understood. The possible development of pharmaceutical or nutriceutical compounds that are based on lactoferrin and can mimic its anabolic bone effects will require a better understanding of lactoferrin's mechanism of action in bone.

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