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Lactoferrin – A Potential Anabolic Intervention in Osteoporosis

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1. Introduction

Osteoporosis or porous bone was first described by Fuller Albright approximately 70 years ago as having “too little bone in the bone”. Bone tissue is maintained throughout life by being continually replaced and in osteoporosis bone resorption exceeds bone formation resulting in bone loss. The majority of current treatments for osteoporosis are anti-resorptive, decreasing osteoclast activity and preventing further bone loss. Therapeutic agents that activate osteoblasts and increase bone formation have the potential benefit of restoring bone rather than only preventing further deterioration, but only a small number of safe anabolic therapies are currently available. Milk is a rich biological fluid that contains many growth factors and provides nutrition at a time of very rapid skeletal growth and development in the neonate, and was therefore considered as a possible source of factors with anabolic effects on bone. Investigations of fractions of whey proteins extracted from milk identified lactoferrin as a bone-active factor. Lactoferrin is an iron-binding glycoprotein which as well as being present in milk is found in other epithelial secretions. It is a pleiotropic factor with potent antimicrobial and immunomodulatory activities, and shows anabolic effects in bone at physiological concentrations. In a number of recent studies in humans and experimental animals dietary lactoferrin supplementation improved bone mineral density, bone markers and bone strength. The current chapter discusses the structure and function of lactoferrin, the bone-effects of lactoferrin *in vitro* and *in vivo*, and the potential use of lactoferrin for the improvement of bone health.

2. Lactoferrin

Lactoferrin is a multifunctional glycoprotein that was originally identified in bovine milk and first isolated from both human and bovine milk five decades ago (Groves et al. 1963). Lactoferrin is produced by mucosal epithelial cells and is present in very high concentrations in milk and colostrum, and in lower concentrations in mucosal secretions, including tears, saliva, nasal and bronchial secretions, bile and gastrointestinal fluids. Lactoferrin is also a major constituent of the secondary granules of neutrophilic leukocytes, and its serum level in healthy subjects is within the range of 1-10 µg/mL (Caccavo et al. 1999).

2.1 Molecular structure

Lactoferrin is a non-haem iron-binding protein which belongs to the transferrin family of iron-transport proteins. It is a highly cationic monomeric glycoprotein with an isoelectric point of about 8.7 (Moguilevsky et al. 1985) and consists of about 690 amino acid residues folded into two homologous lobes, the so-called N-and C-lobes, which are linked by a 10-15 residue alpha helical peptide (Baker & Baker 2005). There is a high degree of homology between lactoferrin from various species with bovine and human lactoferrin sequence identity being 69% (Pierce et al. 1991).

2.1.1 Iron binding and glycosylation

Each of the lactoferrin lobes contains a virtually identical iron-binding pocket, into which a trivalent iron cation (Fe^{3+}) can be reversibly co-ordinated. The metal binding sites are highly conserved for all lactoferrins and transferrins thus far characterised (Baker & Baker 2009). Lactoferrin molecules can exist in several states whereby there is complete, partial (in either one of the two sites) or no occupancy of the two iron-binding sites. Lactoferrin isolated from both human and bovine milk has a low iron saturation, generally reported between 10-25% (Bezwoda & Mansoor 1989). In vitro, iron can be removed from lactoferrin to yield the iron-free or 'apo' form, or alternatively, lactoferrin can be loaded with iron to yield the fully iron-bound or 'holo' form. Although other di- and trivalent transition metal ions such as Mn^{3+} , Co^{3+} , Cu^{2+} and Cr^{3+} and even larger cations such as lanthanides (Smith et al. 1994) can be co-ordinated into the metal binding pocket, iron appears to be the natural ligand as it has optimal co-ordination and a very high binding affinity (Baker 1994). Nevertheless, lactoferrin may have a physiological role in binding trace amounts of other elements as manganese in milk is found exclusively associated with lactoferrin (Lonnerdal et al. 1985).

All lactoferrins are glycosylated, but the number and location of glycosylation sites varies from species to species, and is also tissue specific (Derisbourg et al. 1990). Differentially glycosylated lactoferrins appear to have similar biophysical and functional properties suggesting minimal structural impact of glycosylation (Moguilevsky et al. 1985).

2.1.2 Interactions with other molecules

The highly basic nature of lactoferrin is contributed mainly by surface-exposed N-terminal domains containing clusters of highly basic residues which are capable of binding proteins such as ceruloplasmin (Vasilyev 2010) and osteopontin (Yamniuk et al. 2009). These cationic domains also confer on lactoferrin the ability to bind to many other anionic molecules including heparin, glycosaminoglycans, DNA, and various cell surface molecules (He & Furmanski 1995; Mann et al. 1994; van Berkel et al. 1997).

2.2 Physiological function

2.2.1 Anti-microbial activity

The highly cationic nature of lactoferrin and its high affinity iron binding are implicated in the anti-microbial function of this glycoprotein. Thus, iron sequestration in sites of bacterial infection deprives the bacteria of this essential nutrient, creating a bacteriostatic effect (Gonzalez-Chavez et al. 2009; Jenssen & Hancock 2009). Lactoferrin has bactericidal effect as well, as it interacts directly with anionic molecules on the cell surface, causing cell lysis. In Gram-negative bacteria, lactoferrin interacts directly with LPS, causing its release from the cell wall and increasing the external membrane permeability, which results in cell lysis. In

Gram-positive bacteria lactoferrin damages the cell through direct interactions with lipoteichoic acid and other anionic surface molecules. Iron sequestration and interactions with anionic molecules are also the main mechanisms responsible for lactoferrin's activity against fungus and parasite infections. Lactoferrin has been described as an antiviral agent that affects a broad range of RNA and DNA viruses that infect humans and animals (Gonzalez-Chavez et al. 2009). Although the antiviral mechanisms of lactoferrin have not been well characterised yet, one of the most widely accepted hypothesis is that lactoferrin blocks viral receptors on the cell surface, and in particular heparin sulphate, preventing contact between the virus and the target cell.

2.2.2 Modulation of the immune response and inflammation

Lactoferrin is a modulator of both the innate and acquired immune systems. Following the penetration of a microbe into a tissue, cells of the innate immune system release pro-inflammatory cytokines, including interleukins 1 and 6 (IL-1, IL-6) and tumor necrosis factor-alpha (TNF- α), which increase the permeability of blood vessels enabling the recruitment of circulating neutrophils to the site of infection. The release of neutrophil granule content creates very high local concentrations of lactoferrin. Apart from direct antimicrobial activity, lactoferrin interacts with cells of the innate immune systems as well as with cells of the adaptive immunity; regulating their recruitment, proliferation, differentiation and activation (Legrand et al. 2006; Legrand & Mazurier 2010).

In different experimental systems, lactoferrin acts as either an anti-inflammatory or a pro-inflammatory factor. The anti-inflammatory activity of lactoferrin is attributed to its ability to bind free iron and exogenous proinflammatory bacterial components, such as LPS and their receptors (Legrand et al. 2005). Thus, lactoferrin activity as an iron scavenger prevents the formation of free radicals, which trigger oxidation processes and tissue damage, while binding to proinflammatory molecules inhibits the activation and recruitment of immune cells to the inflamed tissue. An additional mechanism implicated in the anti-inflammatory activity of lactoferrin has been recently described in apoptosis, the process of noninflammatory programmed cell death. Bournazou et al. (Bournazou et al. 2009; 2010) discovered that apoptotic cell of diverse lineages synthesize and secrete lactoferrin, which selectively inhibits the migration of granulocytes but not mononuclear phagocytes. This selective migration allows for the swift phagocytosis of the dying cells by the mononuclear cells without initiating an inflammatory response. Subsequently, lactoferrin was also found to have an inhibitory effect on eosinophil migration (Bournazou et al. 2010). A number of other studies describe the proinflammatory activities of lactoferrin. As a factor that induces inflammation, lactoferrin has been shown to promote cell motility, superoxide production, release of nitric oxide, release of the proinflammatory cytokines TNF- α and IL-8 and phagocytosis (Gahr et al. 1991; Legrand & Mazurier 2010; Shinoda et al. 1996; Sorimachi et al. 1997).

2.2.3 Iron homeostasis and antioxidation

Although lactoferrin is an iron-binding protein and has been shown to influence iron status in pregnant women (Paesano et al. 2009) it is generally thought not to have a central role in iron-transport and homeostasis, unlike the transferrins. Nevertheless it does appear to have some role in iron regulation at local sites of inflammation and infection (Brock 2002) and

iron sequestration is an important part of its role both as an antioxidant and antibacterial agent (Baldwin et al. 1984; Jenssen & Hancock 2009). In respect of iron-binding, lactoferrin is functionally different from transferrin as it retains iron to a much lower pH, giving it a more potent iron-withholding ability (Baker & Baker 2009). Although the antioxidant properties of lactoferrin are generally related to removal of free iron which otherwise reacts with reactive oxygen species (ROS) to form damaging hydroxyl radicals (Matsue et al. 1995; Raghuveer et al. 2002) a recent report suggests that apo- and holo bovine-lactoferrin have equal ability to act as antioxidants by scavenging ROS (Kanwar et al. 2011). This radical quenching ability, akin to antioxidant vitamins, is seemingly iron independent.

2.2.4 Bioactive peptides derived from lactoferrin

Functional cationic peptides with potent antibacterial activity, such as lactoferricin and lactoferrampin, can be derived from the N-terminal domain of lactoferrin by hydrolysis (Bellamy et al. 1992) or synthetic chemistry (van der Kraan et al. 2004), respectively. Lactoferrin can be degraded by digestive enzymes (Brock et al. 1976; Troost et al. 2001) and the functional peptide lactoferricin is likely to be formed in the gut by the action of pepsin. Lactoferrin 'half molecules' consisting of either the N-lobe or C-lobe can be generated by proteolysis or by recombinant technology (Baker & Baker 2005; Kim et al. 2006). These are useful as tools to probe for site-specific functionality or interactions. For example, the anti-herpes virus activity of lactoferrin has been shown to be mediated mainly by the N-lobe (Siciliano et al. 1999) while simple sugars have been shown to interact with the C-lobe through a common recognition site (Mir et al. 2010).

3. The activity of lactoferrin in bone

3.1 Osteoblasts

3.1.1 *In vitro* studies of lactoferrin activity in osteoblasts

Lactoferrin potently induces proliferation of primary osteoblasts and osteoblastic-cell lines and increases osteoblast differentiation at physiological concentrations (Fig 1A) (Cornish et al. 2004; Takayama & Mizumachi 2008, 2009). In 3-week cultures of primary fetal rat osteoblasts lactoferrin dose-dependently increased osteoblast differentiation with increases in bone matrix deposition and the number of mineralized bone nodules formed (Fig 1B) (Cornish et al. 2004). In addition, lactoferrin decreased apoptosis induced by serum withdrawal in primary rat osteoblasts (Fig 1C) (Cornish et al. 2004) and in the human osteoblastic cell line SaOS2 (Grey et al. 2006). These 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 studied in the same *in vitro* assays, such as epidermal growth factor, transforming growth factor- β , parathyroid hormone, amylin or insulin. These factors increase thymidine incorporation in primary osteoblast cultures by only 20 – 30% (Cornish et al. 1999) whereas lactoferrin produces three- to five-fold increments (Cornish et al. 2004). This growth stimulating potency is complemented by the capacity of lactoferrin to substantially reduce osteoblast apoptosis, which again, is much more dramatic than the effects seen with other factors, such as insulin growth factor-1 (IGF-1) which maximally decreases apoptosis by 50% (Cornish et al. 2000) compared to 70% with lactoferrin (Cornish et al. 2004).

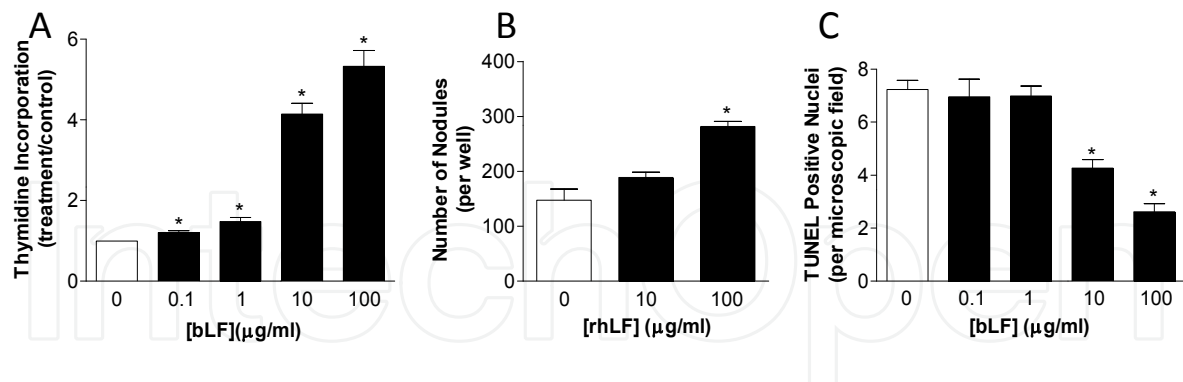


Fig. 1. Lactoferrin stimulates osteoblast proliferation, differentiation and survival *in vitro*

Thus, lactoferrin acts to expand the pool of pre-osteoblastic cells by exerting mitogenic and anti-apoptotic effects, as well as promoting differentiation of precursors to produce a more mature osteoblastic phenotype capable of promoting bone matrix deposition and mineralization.

3.1.2 Local injection model

The activities of lactoferrin on osteoblasts demonstrated *in vitro* are likely to contribute to the potent effects on bone formation seen *in vivo* after administration of lactoferrin, even with a very short-term exposure (Fig 2) (Cornish et al. 2004). The bone growth resulting from local lactoferrin injection is considerably greater than that found previously in response to factors such as insulin, amylin, adrenomedullin and C-terminal PTH-related peptide (Cornish et al. 1996; 1997a; 1997b). 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 (Cornish et al. 1995). This anabolic potency suggests that lactoferrin should be further explored as a therapy for osteoporosis that can restore skeletal strength.

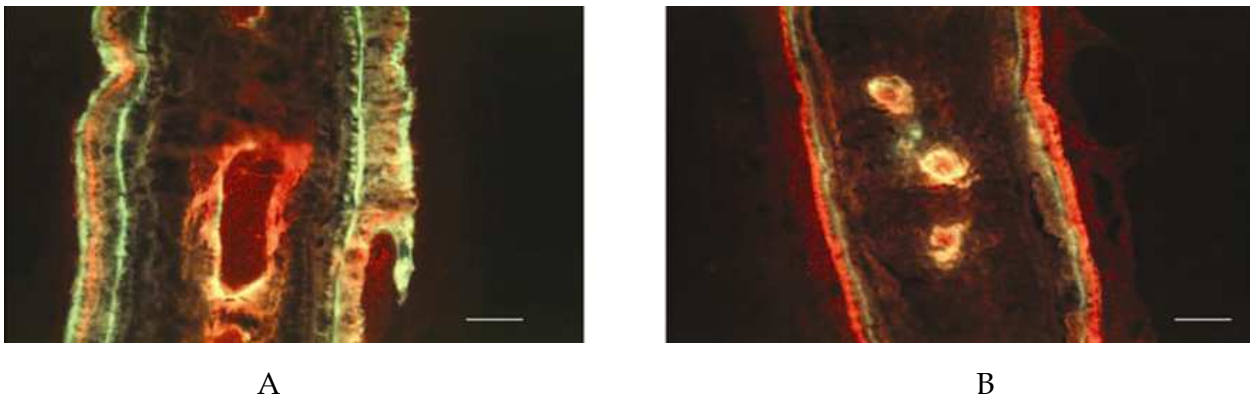


Fig. 2. Photomicrographs of calvariae from animals treated with lactoferrin (A) and vehicle (B) for 5 days. Fluorochrome labels used: green, calcein; red, alizarin. Horizontal bar, 50 μm. (Figure reproduced with kind permission. Cornish J., et al. Lactoferrin is a potent regulator of bone cell activity and increases bone formation *in vivo*. *Endocrinology* 145(9): 2004, 4366-4374. Copyright 2004, The Endocrine Society.)

3.1.3 Signalling pathways activated by lactoferrin in osteoblasts

The downstream pathways activated by lactoferrin are largely unknown, although a number of lactoferrin receptors have been described. A specific lactoferrin receptor was cloned from the human intestine (Kawakami & Lonnerdal 1991) but this receptor is not expressed in all cell types that respond to lactoferrin and we have been unable to detect the mRNA in osteoblastic cells (Naot, unpublished data). Proteins that can bind and induce endocytosis of lactoferrin are nucleolin (Legrand et al. 2004) as well as low-density lipoprotein receptor-related proteins 1 and 2 (LRP1 and LRP2) (Ji & Mahley 1994; Willnow et al. 1992). LRP1 and LRP2 are expressed in osteoblastic cells and LRP1 is at least partially responsible for lactoferrin's mitogenic effects in osteoblasts (Grey et al. 2004). As lactoferrin complexes with LRP1, extracellular signal-regulated kinase (ERK) signalling pathway is upregulated. In addition, lactoferrin upregulates phosphoinositide 3-kinase-dependent Akt signalling but this is in an LRP-independent manner. Lactoferrin's anti-apoptotic activity in osteoblasts is independent of both these two signalling pathways.

In primary osteoblasts, lactoferrin induces a transient, dose-dependent increases in the transcription levels of IL-6, IL-11, the pro-inflammatory factor prostaglandin-endoperoxide synthase 2 (Ptgs2, encoding for the enzyme cyclooxygenase-2, COX-2) and the transcription factor nuclear factor of activated T-cells (Nfatc1). The activity of COX-2 to produce and secrete prostaglandin E2 and the activity of NFATc1 to promote transcription from NFAT consensus elements are also induced by lactoferrin. Moreover, COX-2 and NFATc1 act as mediators of the proliferative effect of lactoferrin in osteoblasts, as inhibition of their activities significantly reduces lactoferrin-induced thymidine incorporation (Naot et al. 2011). Recently, Nakajima et al demonstrated that lactoferrin induces synthesis of angiogenic factors by osteoblasts. In murine osteoblast-like MC3T3-E1 cells and primary murine osteoblasts lactoferrin, purified from milk, increased mRNA expression of vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF2) in a p44/p42 MAP kinase-dependent manner (Nakajima et al. 2011). A summary of some of the signalling pathways activated by lactoferrin in osteoblasts is presented in Figure 3.

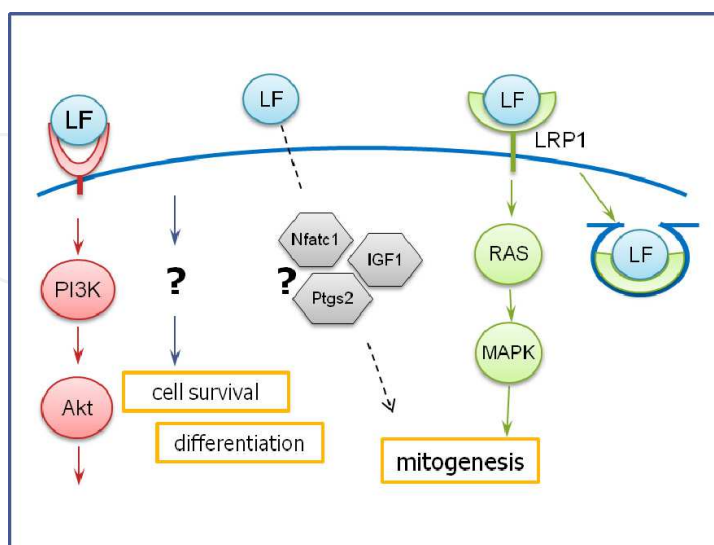


Fig. 3. Mechanisms of action of lactoferrin in osteoblasts. Figure reproduced with kind permission from Springer Science+Business Media: (*Biometals*, Lactoferrin as an effector molecule in the skeleton, 23, 2010, 425-430, Cornish, J. & Naot, D. Figure 1.)

3.1.4 Lactoferrin effects on early stages of osteogenic differentiation

Lactoferrin supports osteogenic differentiation in mesenchymal pluripotent cells whilst reducing adipogenic differentiation. In the pluripotent mesenchymal cell line, C2C12, analyses of expression levels of mRNA and proteins indicated an induction of osteoblastic and chondroblastic differentiation markers and a reduction in myoblastic and adipocytic markers (Yagi et al. 2009). We have identified that lactoferrin reduces adipogenic differentiation in KUSA4b10 cells, a mouse mesenchymal progenitor cell-line capable of developing into adipogenic or osteogenic cell lineages (Fig 4). Lactoferrin has also been found to promote the proliferation and osteogenic differentiation of human adipose stem cells (Ying et al. 2011). The activity of lactoferrin to support osteogenic differentiation whilst reducing adipogenic differentiation could be a promising approach for enhancing osteogenic capacity of cell-based construction in bone tissue engineering.

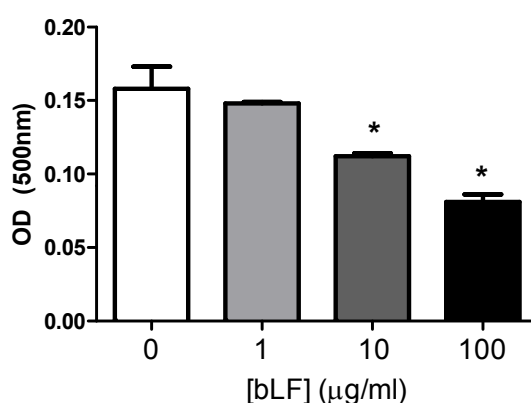


Fig. 4. Lactoferrin reduces adipogenic differentiation in KUSA4b10 cells as measured by Oil Red O release detected in a spectrophotometer at an optical density (OD) of 500nm.

3.2 Osteoclasts

In comparison with actions of lactoferrin on osteoblasts, its osteoclasts effects are strikingly different, in that it produces an almost total arrest of osteoclastogenesis in mouse bone marrow cultures (Fig 5) (Cornish et al. 2004). Reduced bone-resorbing activity was also demonstrated by Lorget et al, who used bovine lactoferrin in a rabbit mixed bone cell culture (Lorget et al. 2002). The mechanisms implicated in the inhibitory effect of lactoferrin on bone resorption are only partially understood. In the rabbit bone cell cultures, lactoferrin inhibited the development of mature osteoclasts by a mechanism independent of the receptor activator of NF- κ B (RANK)/RANK-ligand (RANKL)/osteoprotegerin (OPG) system. In the mouse bone marrow cultures, lactoferrin reduced RANKL expression, which could in part explain the inhibition of osteoclastogenesis, although this was counterbalanced by the effects of lactoferrin to also inhibit expression of OPG (Cornish et al. 2004). As the RANK/RANKL/OPG pathway does not appear to play a major role in mediating the inhibitory effect of lactoferrin on osteoclast formation, the possibility of a direct effect on osteoclasts has been investigated. In RAW264.7 cells, which differentiate into osteoclasts in vitro in the absence of osteoblasts or stromal cells, lactoferrin inhibited RANKL-induced osteoclastogenesis in a dose-dependent manner, demonstrating a direct effect on osteoclasts (Cornish & Naot 2010). This effect of lactoferrin was not blocked by an inhibitor of LRP1, indicating that LRP1 is not the receptor that mediates the direct inhibition of

osteoclastogenesis by lactoferrin (Cornish & Naot 2010). It should be noted that lactoferrin has a capacity to inhibit the survival of progenitor cells in the bone marrow, implying that it might also act earlier in osteoclast development (Hangoc et al. 1991). 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). Thus, lactoferrin inhibits bone resorption by reducing the number of osteoclasts formed from precursor cells.

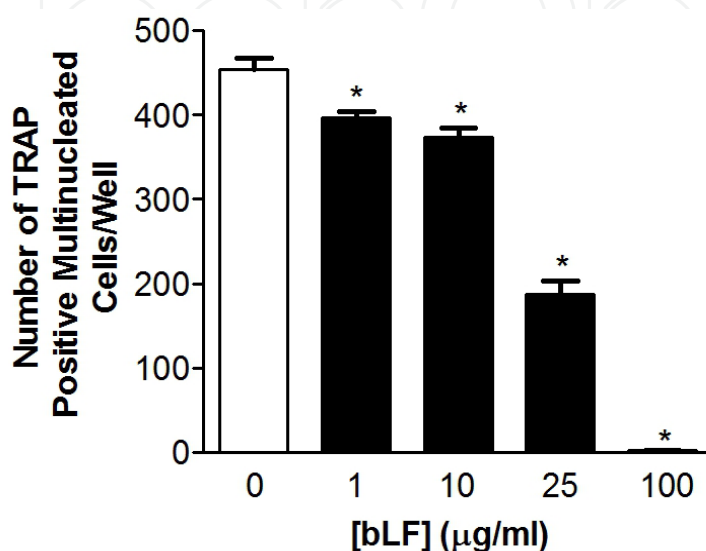


Fig. 5. Inhibition of osteoclastogenesis by lactoferrin in mouse bone marrow cultures

3.3 Structure/Function relationship of lactoferrin's bone activity

Bovine, human and recombinant forms of lactoferrin have comparable ability in stimulating osteoblast proliferation (Cornish et al. 2004). This suggests that glycosylation is not critical to the mitogenic activity as these three forms of lactoferrin are differentially glycosylated. Furthermore, when carbohydrate chains were removed from bovine lactoferrin, the aglyco-form was as potent as the glyco-form (Cornish et al. 2006), confirming that carbohydrate is not a major determinant in the mitogenic activity of lactoferrin in osteoblasts. The activity of lactoferrin on bone cells was also shown to be independent of iron-binding with apo-, native and holo- preparations of bovine lactoferrin giving similar levels of stimulation of proliferation (Cornish et al. 2006). Moreover, replacement of the iron with chromium and manganese, two transition metals of equivalent size, also had no effect. This suggests firstly that the conformational changes induced by iron-binding have no impact on lactoferrin activity, and secondly that bound iron is not essential to this activity.

Further structure/function studies were performed using the N-lobe and C-lobe of bovine lactoferrin (prepared by proteolysis), the N-lobe of human lactoferrin (prepared by recombinant technology), and synthetic bovine lactoferricin peptides (Cornish et al. 2006). The N-lobes of both human and bovine lactoferrin and the C-lobe of bovine lactoferrin all showed osteogenic activity as measured by proliferation of primary rat osteoblasts, but the magnitude of response was less than for the full length molecule (Fig 6A). Interestingly, the bovine C-lobe appeared to have a stronger effect on proliferation than the bovine N-lobe. The bovine lactoferricin peptides (17-31 & 20-30) were both mildly osteogenic (Fig 6B). The N-lobe of human lactoferrin decreased osteoclastogenesis in a dose-dependent manner with an

activity that appeared to be equivalent, on a molar basis, to that of intact recombinant human lactoferrin. In contrast, the C-lobe of bovine lactoferrin has only a weak effect in this assay. The ability of the various lactoferrin lobes and fragments to influence both osteoblast proliferation and osteoclast development suggests that several sites on the lactoferrin molecule might be involved in receptor recognition, binding and stabilisation, or alternatively, more than one receptor might be involved. On a molar basis, the activity of intact lactoferrin on bone cell proliferation was at least 10-fold greater than that of the part molecules, which suggests that a global structure is required for optimal activity. In contrast, the equivalent osteoclastogenic activities of the recombinant human lactoferrin and its N-lobe suggest that this activity might be largely located in the N-lobe. However, further structure/function studies are warranted, as from a therapeutic perspective small active synthetic peptides might present a more attractive option for drug development than the intact lactoferrin molecule.

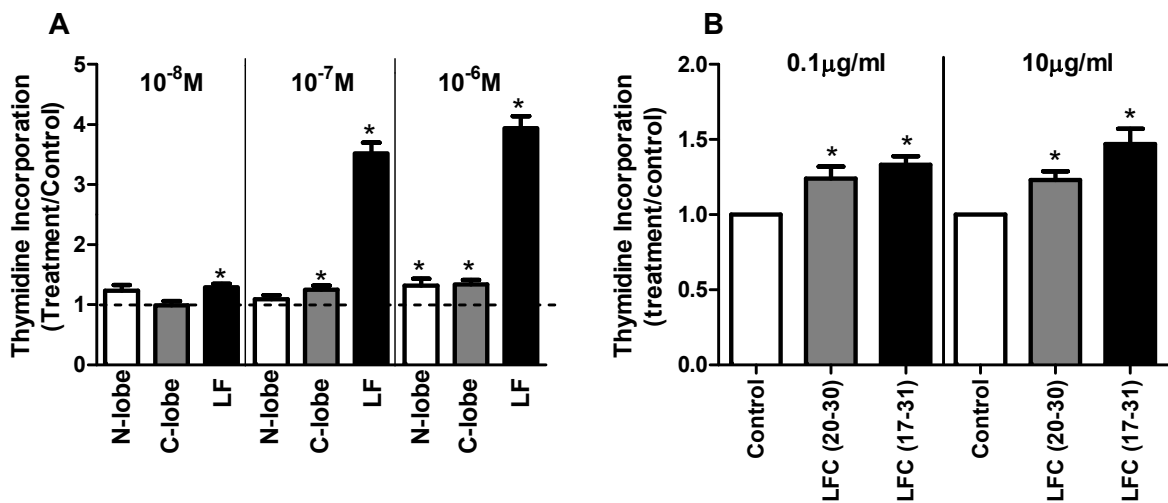


Fig. 6. Thymidine incorporation in primary rat osteoblasts treated with lactoferrin fragments. LF; intact lactoferrin, LFC; lactoferricin.

3.4 Lactoferrin’s activity in bone *in vivo*

A number of recently published studies tested the potential use of lactoferrin for protection against bone loss. The effect of dietary supplementation of lactoferrin on bone was measured using ovariectomized (OVX) rodents as a model for post menopausal bone loss (Blais et al. 2009; Guo et al. 2009; Malet et al. 2011). C3H mice that were either OVX or sham operated, received a control diet or the same diet supplemented with different concentrations of bovine lactoferrin for 27 weeks. Lactoferrin supplementation improved bone mineral density and bone strength, measured as femoral failure load, in a dose-dependent manner (Blais et al. 2009). A study in OVX rats produced similar results. 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 remodelling indicated greater bone formation and reduced bone resorption occurred in rats treated with lactoferrin (Guo et al. 2009). Yamano et al. (Yamano et al. 2010) studied the potential use of lactoferrin for the prevention of alveolar bone destruction associated with periodontitis in an LPS-induced periodontitis rat model. Lactoferrin or liposomal-lactoferrin, which

improved the robustness of bovine lactoferrin to digestive enzymes, were added to the drinking water for 7 days. The study showed that bone resorption stimulated by LPS through activation of TNF- α production and modulation of RANKL/OPG balance in osteoblasts was inhibited by the orally administered lactoferrin. The researchers suggest that liposomal-lactoferrin could be a potent therapeutic and preventive agent for attenuating alveolar bone destruction in periodontitis patients.

In a small clinical study, 38 healthy postmenopausal women were randomized to receive placebo or a ribonuclease-enriched lactoferrin dietary supplement (Bharadwaj et al. 2009). In the lactoferrin-treated group there was a decrease in the bone resorption markers urine deoxypyridinoline (Dpd) crosslinks and serum N-telopeptides and an increase in the bone formation markers bone-specific alkaline phosphatase and osteocalcin, but the results are difficult to interpret due to differences in the levels of markers between the two groups before treatment.

3.5 The expression of lactoferrin in bone and cartilage

Investigations of the expression of lactoferrin in normal fetal and adult bone and cartilage by immunohistochemistry determined that fetal chondroblasts and osteoblasts are positive for lactoferrin immunoreactivity, whereas the corresponding adult cells are negative (Antonio et al. 2010; Ieni et al. 2009a; 2009b; 2011). Bone and cartilaginous specimens from fetuses at 8-34 weeks of gestation were studied. At the eighth gestational week, lactoferrin immunoreactivity was mainly present in the mesenchymal cells forming the periosteum and in chondroblasts; and a lactoferrin signal was also present in immature osteocytes and osteoblasts up to the 18th gestation week. The lactoferrin immunoreactivity decreased considerably by the 24th week, with no expression found in any bone area after the 30th week or in any samples from adult bone (Antonio et al. 2010; Ieni et al. 2011). The expression of lactoferrin in bone and cartilaginous tissue between 8 and 24 weeks of gestation suggests a possible role for lactoferrin as a bone growth regulator in the early phases of the human endochondral ossification.

The expression of lactoferrin was also studied by immunohistochemistry in a large number of tumors of bone and cartilage (Ieni et al. 2009a; 2009b; 2011). About half of all cases of osteocartilaginous tumors were positive, with lactoferrin expression in all giant cell tumors tested, all chondroblastomas, chondromyxoid fibromas and most osteoid osteomas. No lactoferrin immunoexpression was detected in osteosarcomas, chondrosarcomas, ossifying fibromas, osteochondroma and enchondromas. It is possible that lactoferrin expression reflects a less mature phenotype of these tumors, as lactoferrin is absent from normal adult bone and cartilage tissues.

3.6 Lactoferrin as a therapeutic agent

3.6.1 Local delivery

There is much interest in the potential use of lactoferrin as a factor that can act locally in topical applications for regenerative bone therapies and bone tissue engineering. Various biomaterials and biomedical devices have been used to improve delivery and enable sustained release of lactoferrin at the requisite site. Bovine lactoferrin incorporated into a type 1 collagen membrane promoted bone-like tissue formation by MG63 cells which were plated over the membrane (Takayama & Mizumachi 2009) and implantation of biodegradable gelatin hydrogels incorporating lactoferrin into a skull bone defect of rats

resulted in significantly stronger bone regeneration at the defect than was observed in either lactoferrin-free- or low-lactoferrin-treated rats (Takaoka et al. 2011). It was concluded that the sustained release from the gelatin hydrogels enabled lactoferrin to enhance the *in vivo* activity of bone regeneration. A titanium bone plate carrying lactoferrin for treatment of metaphyseal fracture has been patented, primarily as an implant for antibiosis, but such a device could conceivably be used for the promotion of bone repair (Fei et al. 2008). In the same context, a recent technology has been described whereby lactoferrin was coated in thin films onto inert substrates such as silica and biocompatibility assessed for use in applications such as implants (Constantinescu et al. 2009).

3.6.2 Oral delivery

The potential use of lactoferrin as a food supplement that promotes bone health requires experimental evidence showing that it is active when administered orally. Most ingested proteins are degraded into oligopeptides and amino acids in the small intestine and then absorbed as nutrients. The digestion of lactoferrin was studied in adult mice and rats (Fischer et al. 2007; Kuwata et al. 2001). In mice, immunoreactive lactoferrin, measured by ELISA one hour following intragastric intubation of a single dose, was present at the highest concentrations in the stomach, and in lower concentrations in all segments of the intestine: proximal intestine, distal intestine, caecum and large bowel (Fischer et al. 2007). Oral administration of ¹²⁵I-labelled lactoferrin in adult rats, followed by detection of multiple forms of degraded lactoferrin by surface-enhanced laser desorption/ionization (SELDI) affinity mass spectrometry showed that the bioactive fragment lactoferricin (17-42) could survive proteolytic degradation in the small intestine (Kuwata et al. 2001).

Transport of intact lactoferrin from the gut lumen to the circulation has been shown in infants (Hutchens et al. 1989, 1991; Knapp & Hutchens 1994) young calves (Talukder et al. 2002, 2003) and piglets (Harada et al. 1999) suggesting that as the selective transport from the gut is not yet fully developed macromolecules can cross into the circulation. In addition, a number of recent studies demonstrated transport of intact lactoferrin in adult animals and in humans. Fischer et al. (Fischer et al. 2007) found that 10 minutes after the administration of 1mg lactoferrin to adult mice through intragastric intubation, the intact molecule could be detected in the peripheral blood as well as in the liver, kidneys, gall bladder, spleen and brain. Transport of lactoferrin into the circulation has also been shown in groups of Ovx mice that were fed different concentrations of bovine lactoferrin (1-20 g/kg) for 27 weeks. Blood concentrations of immunoreactive lactoferrin of mice that received the bovine lactoferrin-supplemented diets were significantly increased compared to controls and were correlated to the bovine lactoferrin concentration in the diet (Blais et al. 2009).

3.6.3 Functional foods

An important consideration for the use of food systems as vehicles for bioactive delivery is that the bioactive remains active throughout manufacture and shelf-life of the product. Recent work has shown that bovine lactoferrin dosed into stirred yoghurt remained structurally intact and retained its osteogenic activity on primary bone-forming cells up to 21 days after storage of the yoghurt at 4° C (Palmano et al. 2011). In many respects yoghurt is the ideal functional food matrix for bone as it is calcium rich and a popular consumer product.

3.6.4 Lactoferrin preparations with potential use for bone applications

Bovine and human lactoferrin constitute the most studied of the lactoferrins. In general and in spite of some structural differences, bovine and human lactoferrin including recombinant forms appear to have comparable bioactivities. However, some differences between bovine lactoferrin and human lactoferrin have been noted with respect to intestinal receptor recognition (Kawakami & Lonnerdal 1991) and it cannot be assumed that activities are always interchangeable.

The use of lactoferrin as a therapeutic agent requires not only proof of efficacy at the clinical level, but assured safety, consistent quality of supply and appropriate delivery mechanisms. Most studies on the effects of lactoferrin on bone, including clinical trials, have been performed using bovine lactoferrin. Bovine lactoferrin from milk has been available as a commercial isolate for many years (Tomita et al. 2009). It has a 'Generally Recognized As Safe' (GRAS) status from the United States Food and Drug Administration (FDA) and now has widespread acceptance for oral use in humans. Indeed, it has been available for a number of years in Japan and other countries as a dietary supplement and as a functional ingredient in foods such as yoghurt and fortified infant formulae (Wakabayashi & Tasaki 2006). Bovine lactoferrin has been administered orally at doses of 3g/day for one year in a cancer clinical trial, with some positive outcomes and no apparent adverse effects (Tomita et al. 2009).

Equally, recombinant human lactoferrin can be considered for oral application although to date there have been no oral efficacy clinical trials targeted specifically at bone. High expression levels can be achieved in rice (Nandi et al. 2005) and transgenic animals (van Berkel et al. 2002) and recombinant human lactoferrin expressed in both baby kidney hamster cells and rice has been shown to have comparable activity to human lactoferrin and bovine lactoferrin in stimulating proliferation of primary rat osteoblasts (Cornish et al. 2004; Huang et al. 2008). Rice recombinant human lactoferrin was shown to have no toxicity in rats when administered up to 1000 mg/kg body weight/day for 28 days (Bethell et al. 2008a, 2008b) and did not elicit an allergic response in plant-glycan sensitive humans in a limited clinical study (Mari et al. 2008). Moreover it was shown to have beneficial effects as an oral agent in a clinical trial targeted at reduction of diarrhoea in Peruvian children (Zavaleta et al. 2007). No adverse events were reported.

Another potential candidate for bone interventions is Talactoferrin alpha, a proprietary recombinant human lactoferrin expressed in the fungus *Aspergillus awamori* and produced at industrial scale by Aggenix AG (Sanchez et al. 2010). Talactoferrin is currently being evaluated for the oral treatment of several cancer types and Fast Track designation has been granted to Agennix by the FDA for treatment of non-small cell lung cancer (NSCLC) and first-line treatment of renal carcinoma in combination with sunitinib. Placebo-controlled Phase II clinical trials have been successfully completed for NSCLC (Jonasch et al. 2008) and at time of writing two Phase III trials evaluating Talactoferrin in NSCLC patients are ongoing. Talactoferrin appears to have no toxicity, is well tolerated and also appears to be safe for topical applications. It has shown efficacy in the local treatment of diabetic ulcers (Engelmayer et al. 2008) for which it also has Fast Track FDA approval (Sanchez et al. 2010).

4. Conclusion

The positive effects of lactoferrin in bone have been demonstrated *in vitro*; where lactoferrin induces osteoblast proliferation, survival and differentiation and inhibits osteoclast formation, and *in vivo*; where lactoferrin given as a dietary supplement to rat and mice

protects against bone loss associated with oestrogen deficiency. The molecular pathways activated by lactoferrin in bone cells are only partially understood, and it appears that a combination of direct and indirect physiological mechanisms is producing the overall anabolic effect of lactoferrin in bone. Pharmaceutical or nutraceutical use of lactoferrin would require the development of a preparation with assured safety and consistent quality of supply. A better understanding of lactoferrin's mechanism of action in bone would allow for the design of compounds that can mimic its anabolic bone activity, and would be useful in pathological states of reduced bone quality in either systemic or local applications.

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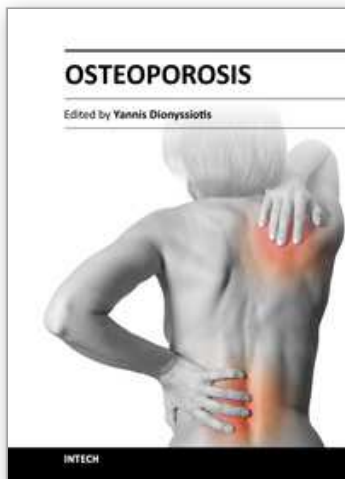
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Osteoporosis is a public health issue worldwide. During the last few years, progress has been made concerning the knowledge of the pathophysiological mechanism of the disease. Sophisticated technologies have added important information in bone mineral density measurements and, additionally, geometrical and mechanical properties of bone. New bone indices have been developed from biochemical and hormonal measurements in order to investigate bone metabolism. Although it is clear that drugs are an essential element of the therapy, beyond medication there are other interventions in the management of the disease. Prevention of osteoporosis starts in young ages and continues during aging in order to prevent fractures associated with impaired quality of life, physical decline, mortality, and high cost for the health system. A number of different specialties are holding the scientific knowledge in osteoporosis. For this reason, we have collected papers from scientific departments all over the world for this book. The book includes up-to-date information about basics of bones, epidemiological data, diagnosis and assessment of osteoporosis, secondary osteoporosis, pediatric issues, prevention and treatment strategies, and research papers from osteoporotic fields.

How to reference

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