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Multifunctional roles of lactoferrin: a critical overview

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Abstract. Lactoferrin (LF) is a member of the transferrin family that is expressed and secreted by glandular epithelial cells and is found in the secondary granules of neutrophils. Originally viewed as an iron-binding protein in milk, with bacteriostatic properties, it is becoming increasingly evident that LF is a multifunctional protein to which several physiological roles have been attributed. These include regulation of iron homeostasis, host defense against a broad range of microbial infections, anti-inflammatory activity, regulation of cellular growth and differentiation and protection against cancer development and metastasis. While iron binding is likely central to some of the biological roles of LF, other activities, including specific interactions with mammalian receptors and microbial components, also contribute to the pleoitropic functional nature of this protein. In this article, recent advances in the understanding of these functions at the cellular and molecular level are discussed.

Key words. Lactoferrin; iron; antimicrobial; cytokine; anti-inflammatory; anti-cancer; bone morphogenesis.

Introduction

Lactoferrin (LF) is an 80-kDa member of the transferrin family of iron-binding glycoproteins [1]. The three-dimensional structure of LF has been precisely defined by X-ray crystallographic analysis, which revealed a globular protein folded into two highly homologous iron-binding lobes [2, 3]. Each of these lobes can bind one ferric ion tightly, but reversibly, with the concomitant binding of a bicarbonate anion [1, 4]. Expression of LF is first detected at the two- to four-cell stage of embryonic development and continues until the blastocyst stage of preimplantation development. LF expression does not resume until the latter half of gestation, where it is detected in neutrophils and in epithelial cells of the developing digestive and respiratory tracts [5]. In the adult, LF is synthesized by glandular epithelial cells and secreted into mucosal fluids that bathe the body surface. Highest levels of LF are detected in colostrum and milk, with lower levels detected in tears, nasal fluids, saliva, pancreatic, gastrointestinal and reproductive tissue secretions [6, 7]. In addition to constitutive expression at the mucosal surface, LF has been shown to be differentially regulated by hormones and transcription factors in a tissue-specific manner. For example, LF expression is under the control of prolactin in the mammary gland, whereas in the reproductive tract, the expression of this protein can be induced by the steroid hormone estrogen [8]. In the hematopoietic system, LF is expressed specifically in developing neutrophils during the myelocyte stage of maturation and is stored in the secondary granules of this cell type [9–12].

Several biological functions have now been described for LF, including iron homeostasis, cellular growth and differentiation, host defense against microbial infection, anti-inflammatory activity and cancer protection [7, 13, 14]. As an evolutionarily conserved iron-binding protein, metal chelation would be expected to be a property central to at least some of LF's functions. Nonetheless, the diverse physiological effects ascribed to LF are undoubtedly also related to its ability to interact with other molecules, including lipopolysaccharide [15, 16], glycosaminoglycans [17] and cell-type-specific receptors that have been identified on a wide range of epithelial and immune cells [13, 14, 18, 19]. The heterogeneous nature of these receptors, coupled with the fact that many have

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other known ligands, including the lipoprotein receptor-related protein [20], a 136-kDa glycophosphatidylinositol (GPI)-anchored intestinal receptor [21], and nucleolin [22], have added to the complexity in elucidating the bona fide physiological roles of this protein that may be receptor-mediated. However, mounting evidence suggests that LF binding to target cells impinges on cellular signalling pathways, including the mitogen-activated protein kinase (MAPK) and the nuclear factor-κB (NF-κB) pathways, resulting in altered gene expression [23–28]. There have also been reports that LF enters the nucleus and activates target genes directly, although further work is warranted in this area [29-31]. This article will provide an overview of the salient functions of LF, in addition to providing an update on our current understanding of the cellular and molecular mechanisms of action that are responsible for the biological activities of LF.

LF and intestinal iron homeostasis

Tight regulation of iron homeostasis is essential, as while iron is required for many metabolic functions in the body, it can be harmful in excess, promoting microbial growth and free radical-induced cellular damage. Iron homeostasis is regulated primarily at the site of absorption in the small intestine in response to body iron requirements [32–34]. It has long been proposed that LF is involved in intestinal iron delivery, although this has been the subject of much debate over the years [7, 35–37]. The high iron bioavailability and abundant concentrations of LF in breast milk suggested that LF may play a role in intestinal iron absorption in the neonate [7, 36]. In addition, LF has been shown to be relatively resistant to proteolysis in the gastrointestinal tract [38], and specific receptors have been identified for LF in the brush border membrane of enterocytes from many species [36]. Furthermore, Caco-2 intestinal cells transfected with a human LF enterocyte receptor demonstrated an increased uptake of LF-bound iron [21]. However, the physiological role of this receptor *in vivo* remains to be established and will likely be clarified further by gene knockout studies in mice.

It is now understood that the recently elucidated divalent metal transporter 1 (DMT-1) pathway is a major mechanism for non-heme iron uptake in the gut [34, 39, 40]. This suggests that milk-derived LF may have functions alternative to intestinal iron delivery. In this regard, it has been proposed that the iron-binding properties and stability of LF function to sequester and remove free iron at the intestinal mucosal barrier [37]. In support of this, it was shown that human infants fed on LF-containing breast milk had a lower rate of iron absorption compared with those fed on LF-free breast milk [41]. Further, recent studies using a genetic mouse model of lactoferrin deficiency (LFKO mice) have demonstrated that LF is not required for iron delivery to the neonate [42]. Rather, comparison of postnatal offspring derived from LFKO intercrosses to wild-type intercrosses (both in a mixed 129/SvEv X C57BL/6 mouse strain) showed that ablation of LF was associated with a mild iron overload. While genetic background may influence the postnatal iron overload phenotype observed in LFKO pups [42], the increased iron levels observed during the suckling period may also be due, at least in part, to the lack of LF in the milk, and more profound effects may be evident under conditions of dietary iron stress. Taken together, this argues against an essential role of LF in intestinal iron delivery and suggests that the iron-binding activity of LF may function primarily to sequester free iron in the gut, thus controlling microbial pathogenesis and iron-induced cellular oxidative damage [37, 43, 44].

Role of LF in the host defense response against microbial infection

The prominent localization of LF in external secretions has long prompted speculation that this protein may play a critical role in maintaining a pathogen-free environment at the mucosal surface [7, 37]. Further, as an abundant neutrophil-derived protein [11], LF can be rapidly mobilized to aid in the host defense response at sites of infection throughout the body. The antimicrobial properties of LF have been well-documented by *in vitro* data and an increasing number of *in vivo* studies [45–47]. It now appears that the ability of this protein to act as a broad-spectrum antimicrobial agent is due to a possession of several distinct antimicrobial properties.

The first antimicrobial property described for LF was bacteriostasis. The strong iron-binding properties of LF, coupled with its relatively iron-free state in body secretions and neutrophils, allow the protein to sequester free iron and maintain an environment refractory to microbial growth [7, 43, 48, 49]. Subsequently it was shown that LF exerted a direct bactericidal activity against pathogens which was independent of its iron sequestration function [50]. *In vitro* experiments by Ellison and colleagues demonstrated that this bactericidal activity was related to the ability of LF to bind directly to the outer membrane of Gram-negative bacteria, causing the rapid release of lipopolysaccharides (LPSs) with an associated increase in membrane permeability and damage [51]. It has been hypothesized that a highly cationic domain located in the N-terminus of LF is responsible for the bactericidal activity of LF, and subsequently it was shown that this isolated peptide, lactoferricin (LFcin), has more potent bactericidal activity than the native protein and is effective against a wide range of microorganisms, including Gram-negative and Gram-positive bacteria, yeast, fungi, viruses and protozoa [52, 53]. However, how LFcin functions in the context of the intact protein and/or whether

substantial amounts of this peptide are released at sites of infection *in vivo,* remains to be established.

In the past decade or so, several additional properties have been described for LF which likely contribute to its versatility as a physiologically important antimicrobial agent in mucosal host defense. A recent elegant study by Singh and colleagues demonstrated that iron sequestration by LF was inhibitory to bacterial biofilm formation [54]. LF was shown to prevent *Pseudomonas aeruginosa* biofilm formation in continuously cultured mammalian flow cells by stimulating a specialized bacterial motion called twitching. Twitching prevents the bacteria from attaching to the surface of the mammalian cells and forming the microcolonies that ultimately give rise to biofilm formation. Although dependent on the iron-binding properties of LF, the anti-biofilm activity was observed using extremely low concentrations of LF (0.02 mg/ml), which is fivefold less than that required to inhibit the growth of this bacterium by bacteriostatic mechanisms [54]. Interestingly, LF is proteolytically degraded in the airway secretions of cystic fibrosis (CF) patients, who are particularly susceptible to chronic infection and biofilm formation with *P. aeruginosa* [55]. Further, *in vitro* studies confirmed that proteolytic degradation of LF resulted in a loss of its anti-biofilm activity [56]. Collectively, these studies suggest that the anti-biofilm property of LF may have physiological relevance and contribute to the host defense response against *P. aeruginosa* and possibly other bacterial biofilm infections at the mucosal surface.

Studies have also demonstrated that LF can protect epithelial cells against microbial infection by inhibiting intracellular invasion by pathogenic bacteria, presumably by binding to surface bacterial and/or mammalian proteins and blocking their adhesion to host cells [57–59]. This mechanism appears to also account for most of the anti-viral activity described for LF. In this regard, LF has been shown to inhibit the initial stages of infection by several viruses, including human immunodeficiency virus, human hepatitis C virus, rotavirus and respiratory syncytial virus, by binding directly to the virus particles and/or binding to docking or receptor sites for the virus on target mammalian cells [60–62].

Intriguingly, LF may protect against microbial pathogenesis by a recently uncovered proteolytic activity of the protein. LF has been shown to degrade and inactivate proteins that are required for bacterial colonization by enteropathogenic *Escherichia coli*, *Shigella flexneri* and *Haemophilus influenzae* [63–66]. This activity was blocked by serine protease inhibitors, and subsequent studies characterized a serine protease catalytic domain in the N-terminal domain of the LF protein that can cleave arginine-rich bacterial sequences [67]. While the significance of this proteolytic activity to microbial protection *in vivo* remains to be established, the highly conserved nature of the proteolytic site between LF species strongly suggests that it may contribute to the physiological host defense response of LF [67].

Evidence suggests that LF may also protect against microbial pathogenesis indirectly through the stimulation of the host immune system. For example, the upregulation of T helper type 1 (TH1) immune responses were associated with *Staphylococcus aureus* clearance in lactoferrin transgenic mice [68] and a T cell-dependent stimulation of natural killer (NK) cell activity was shown to contribute to the antiviral effect of LF against murine cytomegalovirus in a mouse model of infection [69].

Finally, it is noteworthy that, although the multitude of antimicrobial activities ascribed to LF suggest that it plays a critical role in the protective innate immune response to microbial infection, in certain instances LF can be rendered inactive by the invading pathogen or can even enhance microbial pathogenicity. For example, the pneumococcal surface protein A of *Streptococcus pneumoniae* can bind to LF and protect the bacteria from the killing action of LF [70], whereas *Neisseriaceae* and *Moxarella* species may utilize LF-bound iron required for their growth by synthesizing specific LF receptors that can bind and extract iron from LF [71].

Anti-inflammatory role of LF

In addition to controlling bacterial burden by direct antimicrobial action, evidence suggests that LF may limit the inflammation associated with microbial challenge. In support of this, animal studies have shown that LF administration protects against gastritis induced by *Helicobacter pylori* [72], gut mucosal integrity induced by LPS challenge [73], and endotoxemia and lethality in response to systemic challenge with *E. coli* or LPS [74–76]. Both *in vitro* experiments in mononuclear cells and *in vivo* studies in mice suggest that the protective effect of LF may involve an inhibition of production of several pro-inflammatory cytokines, including tumor necrosis factor alpha (TNFα), interleukin-1β (IL-1β) and IL-6 [27, 77–79]. This may be mediated in part by the ability of LF to sequester molecules that interact with the Toll-like receptor signalling cascade that plays a key role in the ensuing host inflammatory response to microbial infection [80]. In support of this, LF has been shown to directly bind and attenuate the immunostimulatory response of LPS, soluble CD14 (sCD14) and unmethylated CpG bacterial DNA [16, 19, 81]. Finally, *in vitro* studies in monocytic cells suggest that the anti-inflammatory activity of LF in response to LPS challenge may also involve inhibition of pro-inflammatory cytokine synthesis following LF translocation to the nucleus, where it prevents NF-κB activation [27].

It is becoming increasingly evident that the anti-inflammatory role of LF extends beyond attenuation of microbi-

al-induced inflammation. LF is known to be upregulated in inflammatory disorders, including neurodegenerative disease [82–84], inflammatory bowel disease [85–87], allergic skin and lung disorders [88, 89], and arthritis [90, 91]. Moreover, a number of animal studies have shown that LF administration can alleviate experimental inflammation in these tissues. For example, LF protects against chemical and IL-1β-induced cutaneous inflammation in humans and mice [92–95], chemically induced inflammatory bowel disease in rats and mice [96–98], non-steroidal anti-inflammatory drug (NSAID)-induced intestinal injury [99] in rodents and inflammation in a rat model of rheumatoid arthritis [100]. In many cases, this protection was associated with a decrease in proinflammatory cytokines, in particular TNF-α and IL-1β and/or an increase in anti-inflammatory cytokines, including IL-10. While mechanistically poorly understood, the ability of LF to interact with specific receptors on many immune cells, including neutrophils [101], monocytes [102], macrophages [103] and lymphocytes [104], in addition to epithelial cells [13, 21, 105], suggests that the anti-inflammatory activity of LF may be a result of a direct effect on modulating cytokine production by these cells via receptor-mediated signalling pathways.

Various other mechanisms whereby LF may downregulate the inflammatory response have also been suggested, including prevention of iron-catalyzed free radical damage at sites of inflammation [106] and abrogation of the late phase airway obstruction and hyperresponsiveness in a sheep model of allergic asthma secondary to tryptase destabilization [107].

Protection against cancer development and metastasis

A growing number of rodent studies have demonstrated a protective effect of LF against chemically induced carcinogenesis, tumor growth and/or metastasis in several organs, including the esophagus, tongue, lung, liver, colon and bladder [108–121]. Moreover, it appears that like many of the biological functions of LF, the anti-cancer role of this protein may be multifaceted. A direct effect on tumor cell growth was first suggested by the observation that LF and a splice variant thereof are downregulated or absent in many cancer cell lines and in experimental tumors [122–127]. This was further supported by tissue culture studies with human breast carcinoma cells as well as head and neck cancer cell lines demonstrating that LF blocked the G_1 to S transition of the cell cycle. The negative effect on cellular proliferation appears to be due to LF-induced alterations in the expression and/or activity of critical cell cycle regulatory proteins including the Cdk inhibitors p21 and p27, which may be mediated in part by modulation of the Akt and MAPK pathways [23, 128]. *In vivo* studies also suggest that the inhibition of tumor cell growth by LF may be related to the ability of this protein to induce apoptosis of cancer cells by activating the FAS signalling pathway in cancerous cells [129, 130]. Interestingly, LF has recently been shown to activate the NF-κB signalling cascade in HeLa cervical carcinoma cells, which resulted in upregulation of the tumor suppressor protein p53 and its target genes, mdm2 and p21, although the implications for cellular growth were not addressed in this study [28].

Accumulating evidence also suggests that immunomodulation may be critical to the anti-cancer function of LF. *In vitro* studies have shown that LF stimulates the production and/or activation of several immune cells, including lymphocytes and NK cells [131–133], in addition to increasing the target cell sensitivity to NK lysis [134]. An early study by Bezault et al. implicated enhanced NK cell activity as a mechanism by which LF prevents carcinogenesis. In these experiments, it was shown that intraperitoneal administration of human LF to mice inhibited the growth of solid tumors and prevented against lung metastasis by melanoma cells, an effect which was lost upon depletion of the NK cell function using antibody blocking experiments [108]. In addition, the inhibitory effect of LF on tumor cell growth was reported to be greater in immunocompetent compared with immunodeficient mice [109]. Moreover, it has now been shown that the protective effect of oral administration of LF in several rodent cancer models is associated with enhancement of the local intestinal mucosal immune response. In this regard, upregulation and/or enhanced activation of NK cells, $CD4^+$ T lymphocytes and $CD8^+$ T-lymphocytes were observed upon LF administration [110, 113, 121]. Interestingly, enhancement of the systemic immune response was also observed despite a reported low absorption of this protein from the intestine [110, 113, 120, 121].

Recent studies suggest that the mechanism underlying the immunoprotective effects of LF against cancer cell development may be mediated in part by interleukin-18, a cytokine with pleiotropic effects on immune cell activation and function [135]. In this regard, it has been shown that LF strongly upregulates IL-18 expression in intestinal epithelium, possibly secondary to the activation of caspase-1, which is required for enzymatic cleavage of the active form of IL-18 [120, 121, 136]. The presumption is that secreting IL-18 locally in the intestine and into the serum may be central to the coordinated upregulation of the mucosal and systemic immune responses [114, 137]. Although the molecular pathway leading to caspase-1/interleukin-18 activation is unknown, conceivably this may occur by LF signalling through specific receptors on intestinal and/or local immune cells to regulate cytokine production. LF was also shown to inhibit tumor-initiated angiogenesis *in vitro* and *in vivo*, which may relate to the anti-angiogenic properties of IL-18 [111, 112, 138].

LF as a regulator of organ morphogenesis

One of the most recent novel activities described for LF is its regulatory function in bone morphogenesis. LF was shown to prevent bone resorption in a rabbit mixed bone cell culture [139]. Subsequent experiments using cultured rodent tissue and organ cultures showed that LF promotes the growth and development of osteoblast cells by stimulating proliferation and decreasing apoptosis [140]. In addition, LF was shown to enhance osteoblast differentiation and inhibit osteoclastogenesis. The growth-promoting effects demonstrated for LF were far more potent than the response seen by established bone growth factors, including epidermal growth factor. Importantly, the anabolic effects on bone growth were substantiated by *in vivo* studies where subcutaneous administration of LF (4 mg daily for 5 days) to mice resulted in a fourfold increase in bone mass [140]. In a follow-up study, it was shown that the mitogenic response of LF in osteoblasts is mediated in part by binding and signalling through the low-density lipoprotein receptor-related protein-1 (LRP-1) [26]. The molecular events downstream of LF interaction with LRP-1 that result in osteoblast proliferation have begun to be unravelled by the finding that the p42/p44 MAPK signalling pathway is involved. Interestingly, while LRP1 also mediates LF endocytosis into these cells, uptake of LF is not required for the mitogenic function of this protein, as LF promotes the growth of osteoblasts under conditions where endocytosis is abrogated. Although the physiological relevance of these findings during normal bone development is unknown, these novel findings suggest that LF administration may have potential therapeutic implications for osteoporosis treatment [26].

LF has also been demonstrated to have mitogenic effects on other cell types, including rat and human enterocytes [141–144], B and T lymphocytes [104, 145] and macrophages [146]. However, the involvement, if any, of LRP1 and/or the MAPK pathway in transducing the proliferative signal in these cell types has not been addressed.

Summary and future perspectives

It is now emerging that LF is a multifunctional protein that impinges on several physiological and pathological processes, including iron homeostasis, organ morphogenesis and host defense against infection, inflammation and cancer. It appears that for each of the functions described for LF, several distinct mechanisms of action are involved which may act in unison to augment the biological effect of this protein *in vivo*. One of the emerging themes underlying the morphogenic, anti-inflammatory and cancer protective functions of this protein is the ability of LF to regulate cellular signalling pathways. Presumably, this is mediated in large part by the ability of LF to bind to a wide range of epithelial and immune cells. However, the cellular localization and downstream molecular events following LF engagement with these receptors is, for the most part, still largely unknown.

Originally supported by *in vitro* data, the functions ascribed to LF are now being by an increasing number of *in vivo* studies. Nonetheless, these studies do suggest that this protein may have potential therapeutic use in microbial, inflammatory and cancer disease prevention and/or treatment. Finally, the availability of genetic models of LF ablation and tissue-specific LF transgenic mice will clearly be invaluable tools to rigorously investigate the essential physiological and/or therapeutic functions of this protein *in vivo*. However, caution must be exected in the interpretation of some of these findings, in particular where high doses of non homologous LF of unknown purity was used.

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