THE BIOLOGICAL PROPERTIES OF LACTOFERRIN

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THE BIOLOGICAL PROPERTIES OF LACTOFERRIN

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Abstract Lactoferrin (LF) is an iron-binding glycoprotein from the transferrin family, which has been identified in most biological fluids as secretions from exocrine glands and the content of specific granules of neutrophils. It has been reported to have numerous functions. Due to antimicrobial and anti-inflammatory activity, the lactoferrin plays significant role in host defense against infection and extreme inflammation. Recent studies have also demonstrated that LF can protect against cancer in experimental animals and has anticarcinogenic activity in many human tumors. At the cellular level, LF modulates the proliferation, differentiation, maturation, activation, migration and function of immune cells. This review presents the multifunctional roles and specific beneficial properties of lactoferrin.

Key words lactoferrin, antimicrobial activity, immunomodulatory effect

Introduction

Lactoferrin (LF) is a non-heme iron-binding protein of the transferrin family with a high affinity for iron, even a 2-fold higher than transferrin (Ward et al., 1999; Ward, Paz, Conneely, 2005; Adlerova, Bartoskova, Faldyna, 2008).

Lactoferrin was first isolated from cow's milk in 1939 (Sorensen, Sorensen, 1939), and the its presence in human milk was determined in 1960 by three independent research centers (Groves, 1960; Johanson, 1960; Montreuil, Tonnelat, Mullet, 1960). Further studies aimed at determining the sites of lactoferrin synthesis in the body. LF expression has been shown in the cells of the preimplantation mouse embryo at the stage of 2 to 4 cells, persisting until the blastocyst stage. It appears again in the second half of pregnancy, where it is expressed in neutrophils and epithelial cells of the developing gastrointestinal and respiratory systems (Ward et al., 1999). In an adult organism,

lactoferrin is synthesized by glandular epithelial cells and is released into the mucosal fluids that bathe the surface of organs. Its maximum concentrations are found in colostrum and milk, and lower levels in secretory fluids such as tears, saliva, nasal and bronchial secretions, and in the exocrine secretions of pancreas, gastrointestinal tract, and the genital system (Adlerova et al., 2008; Ward, Uribe-Luna, Conneely, 2002). Its presence has been confirmed in the specific granules of neutrophils (Ward et al., 2002) and lactoferrin synthesis takes place during granulopoiesis at the myelocyte stage.

Structure and function of lactoferrin

Human lactoferrin is a positively charged protein composed of a single polypeptide chain comprising 703 amino acids, folded into two symmetrical globular lobes - N and C lobes. Each lobe is organized into two domains (N: N1 and N2; C: C1 and C2 domains) connected by a hinge region containing a three-turn α-helix (Figure 1). Both lobes show 33–41% homology in structure. Each lobe has one binding site for iron ions (Fe^{+2} or Fe^{+3}), and one or more potential glycosylation sites, depending on the species from which LF is isolated. Depending on its form, the molecular weight of LF varies between 76 and 80 kD (Levay, Viljoen, 1995; Siqueiros-Cendón et al., 2014). The degree of iron saturation determines the spatial structure of LF, which occurs in two forms: apolactoferrin (apo-LF), with low iron saturation, and iron-rich hololactoferrin (holo-LF) (Baker, Baker, 2005).

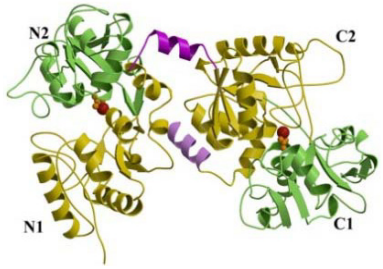

Figure 1. Structure of lactoferrin (According to Berlutti et al., 2011)

The commercially available bioactive recombinant human lactoferrin (rhLF) has three different forms with different levels of iron saturation; apo-rhLF (iron-free <10%), pis-rhLF (partially iron-saturated, at ~50%) and holo-rhLF (>90% saturation) (Nandi et al., 2002; Nandi et al., 2005; Amini, Nair, 2013). The lactoferrin affinity for iron depends on pH and increases when pH becomes slightly acidic (Kanwar et al., 2015). Partial iron saturation of lactoferrin (at 15–20%) occurs naturally in the body. LF shows high structural homology irrespective of the mammalian species from which it was isolated (Małaczewska, Rotkiewicz, 2007).

Lactoferrin has been extracted from human colostrum (human lactoferrin, hLF); goat (goat lactoferrin, gLF) with high homology with hLF; camel (camel lactofferin, cLF) and cow (bovine lactoferrin, bLF) (Kanwar et al., 2015). Researchers have established the functions of lactoferrin originating from various sources (Table 1), with many recent studies bringing new discoveries about its role. LF has a wide range of-physiological properties, showing immunomodulatory, anti-inflammatory, antibacterial, antiviral, antifungal, antiparasitic, anticancer activities,

outstanding osteogenic activity and promoting formation of new blood vessels (Figure 2). Associated with the mucosal tissue, LF is an important component of the innate immune system. It exhibits bacteriostatic and bactericidal properties against Gram-positive (+) and Gram-negative (-) bacteria. One of its basic and well-known functions is iron transport. Produced by specialized cells, e.g. in kidneys, LF exhibits both antibacterial and antioxidant effects, protecting against urinary tract infections. Here, the mechanism of action consists in controlling and reducing the concentration of free iron available to bacteria in the urinary system (Małaczewska, Rotkiewicz, 2007).

Table 1. Lactoferrin: various sources, functions and roles (According to Kanwar et al., 2015)

Lactoferrin Source	Action	Functional Role
Human Lactoferrin	Anti-microbial	Effective against *Streptococcus*, *Salmonella*, *Shigella*, *Staphylococcus* and *Enterobacter*. Enhances the host immune system.
	Anti-cancer	Diagnostic marker.
Goat Lactoferrin	Ongoing research	Still novel and further studies need to be conducted.
Camel Lactoferrin	Anti-viral	Inhibits infection by Hepatitis C and B virus. It has hepatoprotective effect.
	Anti-diabetic	Potential therapeutic molecule in targeting both type 1 and type 2 diabetes. More work needs to be done.
Bovine Lactoferrin	Anti-cancer	Anticancer activity against colorectal cancer and lung cancer.
	Anti-microbial	Effective against oral candidiasis, influenza virus pneumonia and skin infections due to herpes virus. Enhances host immune response. Anti-inflammatory.

The regulation of LF concentration involves macrophages and monocytes with high-affinity receptors for LF, enabling them to quickly remove excess LF from the circulation. The receptors bind iron, transforming it into ferritin, while the LF molecule is degraded.

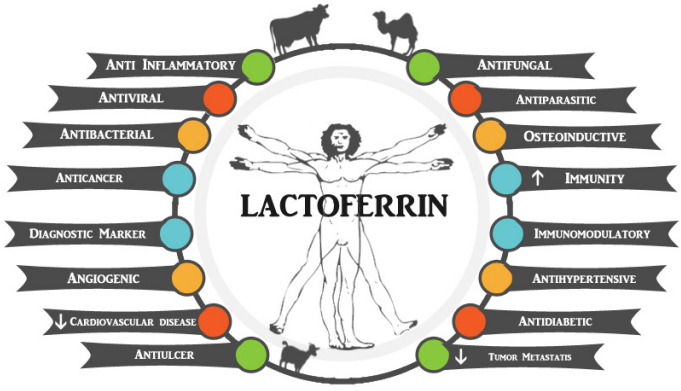

Figure 2. Lactoferrin and its functions

The antimicrobial properties of lactoferrin

The bactericidal effect is a very important property of LF, making it an interesting alternative in antibiotic resistance. It has been shown that lactoferrin is an effective antimicrobial agent (Li, Tan, Vlassara, 1995; Artym, 2010).

The lactoferrin's antibacterial activity is attributed to its ability to sequester iron, an element necessary for the growth and proliferation of microorganisms in body fluids (Artym, 2010). Inhibition of bacterial proliferation by lactoferrin via free iron chelation is one of its earliest discovered functions. Its antibacterial activity was determined by *in vitro* studies in which 0.5% solution of the purified lactoferrin obtained from human milk, free of immunoglobulin, lysozyme and transferrin, were placed on the surface of gel with two species of bacteria, *Staphylococcus albus* and *Staphylococcus aureus*. LF was shown to inhibit the growth of *S. albus*, and introduction of ionized iron to the solution of lactoferrin neutralized that effect (Masson, Heremans, Prignot, Wauters, 1966).

Lactoferrin also has a bactericidal effect not related to the binding of iron. It has an ability to influence bacteria directly, thanks to its specific structure with a highly positively charged N-terminal region (Redwan, Uversky, El-Fakharany, Al-Mehdar, 2014). Acting on the cell walls of Gram positive bacteria, LF is capable of disintegrating them, increasing their permeability, and in consequence inducing cell-death. LF binds to lipopolysaccharide (LPS), an integral part of the walls of Gram-negative bacteria, which leads to their disintegration. Experimental studies have demonstrated that the LF's bacterocidal effect depends on its concentration (Drago-Serrano, Garza-Amaya, Luna, Campos-Rodríguez, 2012). A direct contact of lactoferrin with pepsin in the stomach leads to the digestion or hydrolytic degradation. Thus generated lactoferricin has more potent antibacterial effect and broader spectrum of action than native LF. The inhibitory effect on micro-organisms is obtained at a low dose (0.5–500 mg/mL) (Małaczewska, Rotkiewicz, 2007). LF also shows a synergistic effect in combination with antibiotics. The mechanism consisting in increasing the cell wall permeability facilitates the penetration of the antibiotic into the cytoplasm of the target cell. This results in a faster and more effective chemotherapeutic action (Farnaud, Evans, 2003).

Lactoferrin is also used in the treatment of periodontal disease, thanks to its bacteriostatic action against plaque-forming bacteria, such as *Streptococcus mitis*, *Streptococcus gordoni*, *Streptococcus salivarius* and *Streptococcus mutans* (Kanwar et al., 2015).

Lactoferrin is one of the proteins present in saliva, at 1.23 mg/L of gingival crevicular fluids (GCF) and 8.96 mg/L in unstimulated and 7.11 mg/l in the stimulated saliva. LF concentration in the gingival crevicular fluid depends on the volume of secreted saliva, but also upon the pathological condition in the oral cavity. It has been shown in the locally collected samples from the oral cavity of patients with gum disease, concentration of LF is increased to 63 ng/site, while in those with periodontal disease it increased to 90 ng/site, compared to the levels in healthy subjects (36 ng/site/site) (Wei et al., 2004; Berlutti et al., 2011). The significance of LF in periodontal diseases is emphasized by experimental studies. In tests on mice with lactoferrin knockout ($LFKO^{-/-}$) and alloxan-induced diabetes, the animals were more susceptible to periodontitis induced by *Aggregatibacter actinomycetemcomitans* (Alabdulmohsen, Rozario, Markowitz, Fine, Velliyagounder, 2015).

Periodontal diseases are associated with inflammation in tissue surrounding the tooth, due to the accumulation of subgingival plaque formed mainly by Gram-negative bacteria (Wakabayashi et al., 2010; Berlutti et al., 2011). A new method of treating periodontal disease uses bovine LF (1) inhibiting the inflammatory process by binding free iron ions, (2) binding to the surface of the bacteria (Berlutti et al., 2010; Wakabayashi et al., 2010; Latorre, Berlutti, Valenti, Gessani, Puddu, 2012), and (3) inhibiting the growth of biofilm (Chen, Wen, 2011).

Bacteriostatic properties of LF are confirmed by clinical trials. The frequent problem of halitosis (oral malodor) caused by bacterial metabolism (e.g. inhabiting the oral cavity) is found in approximately 50% of patients in the world, and in 90% the etiology is related to processes in the oral cavity (Armstrong, Sensat, Stoltenberg, 2010). It is accompanied by the presence of plaque and tartar, periodontal diseases, such as periodontitis, and infections involving dentures, tooth decay, mouth ulcers and ulceration (Chomyszyn-Gajewska, Skrzypek, 2013). In randomized trials, patients with halitosis were given single oral dose of a commercial drug (*Morinaga Orabarrier, Morinaga Milk Industry Co., Ltd.,* Tokyo, Japan), a tablet containing 20 mg of lactoferrin, 2.6 mg lactoperoxidase, and 2.6 mg of glucose oxidase. As early as 30 minutes after administration of the tablet the malodour was inhibited (Nakano, Shimizu, Wakabayashi, Yamauchi, Abe, 2016).

Antiviral properties of lactoferrin

The antiviral effect of LF consists in inhibiting the replication of viral DNA and RNA. One of the mechanisms of action, confirmed in experimental models, is its protective effect against virus-free cells. LF has also been observed to bind directly to molecules in the structures of viruses such as HSV, HIV and HCV (Välimaa, Tenovuo, Waris, Hukkanen, 2009; Berlutti et al., 2011). Another mechanism of the antiviral action of LF is its ability to block the host's cell surface receptors. The LF's affinity for glycosaminoglycans results in blocking the virus binding sites in the initial phase of infection (Redwan et al., 2014). This prevents the use of surface molecules as specific receptors or co-receptors for the different types of viruses and prevents viral fusion. This mechanism has been described, inter alia, in HBV, HPV, HSV, and HIV (Berlutti et al., 2011; Wakabayashi, Oda, Yamauchi, Abe, 2014). It has also been shown that the effect of iron-free apolactoferrin on some viruses was greater than that of holo-lactoferrin (Małaczewska, Rotkiewicz, 2007).

Antifungal properties of lactoferrin

Antifungal properties of LF are related to its ability to damage fungal cell membranes and alter their permeability, and also to iron chelation (Wakabayashi et al., 2000). These effects have been confirmed *in vivo* in mice, in which hLF effectively protected against experimental oral infection with *C. albicans* (Velliyagounder, Alsaedi, Alabdulmohsen, Markowitz, Fine, 2015).The native human lactoferrin showed a higher activity against *Candida*, as measured by the minimum inhibitory concentration (MIC), than the form with high iron-saturation (Grammatikova et al., 2010).

Antiparasitic properties of lactoferrin

Lactoferrin also exhibits a beneficial effect in parasitoses. Although it does not inhibit the entry of parasites into the body, it does inhibit their growth (Cintra, Silva-Filho, De Souza, 1986). The ability of iron chelation blocks the ion uptake by parasites, the main mechanism directed against *Pneumocystis carinii.* It has been observed that LF preincubated with the sporozoites of *Toxoplasma gondii* greatly reduces their infection ability. The mechanisms of the protective effect of LF have not been fully explained. It is believed that the observed antiparasitic effects are associated with the activation of macrophages, as well as with the direct effect on membrane integrity of the parasites and positive interactions with host tissues (Farnaud, Evans, 2003; Małaczewska, Rotkiewicz, 2007).

Anti-inflammatory properties

A multifunctional protein, lactoferrin also exhibits the ability to modulate the body's inflammatory response, affecting the immune system in different ways. LF enhances proliferation, differentiation, and causes the activation of immune cells. It affects the mechanisms of the innate response, by influencing the activation of the complement system, an increase in NK cell activity, increase in the phagocytic ability of monocytes and their enhanced cytotoxicity (Legrand, Mazurier, 2010). LF also reduces the amount of produced proinflammatory cytokines (TNFalpha) and interleukins IL-1 and IL-6 (Haversen et al., 2002). It affects hemostasis by reducing the time of clot formation, and prevents platelet aggregation (Brock, 2002). Its anti-inflammatory properties are also linked to the ability to bind components of bacterial cell walls (LPS) as well as the specific receptors (Morgenthau, Beddek, Schryvers, 2014). Lipopolysaccharides, potent mediators of the inflammatory response, cause the activation of leukocytes which results in the hyperproduction of free radicals. Iron is a catalyst for these reactions (Actor, Hwang, Kruzel, 2009). Thanks to its chelating properties, LF can reduce the indirect effects of reactive oxygen species at inflammation sites and thus protects the tissue against damage (Ward et al., 2005). Infection results in neutrophil degranulation, and LF released from their specific granules has the ability to quickly inactivate LPS, thereby protecting the tissue against damage (Baveye, Elass, Mazurier, Legrand, 2000; Ammons, Copié, 2013). Anti-inflammatory properties of LF have also been confirmed by *in vivo* tests in animal models. Oral administration of LF resulted in reducing the symptoms of inflammation in mice and the subsequent occurrence inflammation in experimentally induced colitis (Haversen, Baltzer, Dolphin, Hanson, Mattsby-Baltzer, 2003).

Anticancer properties

Lactoferrin prevents from developing chemically-induced tumors. This effect has been confirmed in studies conducted on laboratory rodents (Tsuda, Sekine, Fujita, Ligo, 2002). LF decreases the incidence of metastases in experimental mice diagnosed with the disease (Wolf, Li, Taylor, O'Malley, 2003). Fujita suggests that the inhibition of tumor growth may be associated with tumor cell apoptosis via the activation of the Fas pathway (Fujita, Matsuda, Sekine, Iigo, Tsuda, 2004). Studies on human and mouse cell lines have shown that the administration of recombinant human lactoferrin can inhibit the growth of head and neck squamous cell carcinoma (HNSCC), via a direct impact on the development of cells, and also through systemic immunomodulation (Wolf et al., 2007). A similar effect has been observed in the case of glioblastoma. Furthermore, the combined administration of lactoferrin and temozolomide enhances the effect of chemotherapy both in vitro and in vivo (Arcella et al., 2015).

Lactoferrin and immunity

As mentioned above, lactoferrin is present in all body fluids, and its iron-free form is stored in specific granules of neutrophils. During inflammation, LF is released from the granules, which increases its concentrations at the inflammation site from 0.4–2.0 μg/mL to 200 μg/mL, playing a central role in response to inflammation (Farnaud, Evans, 2003). Its expression at the RNA and protein levels has also been demonstrated in cells of the distal collecting ducts of the medulla. LF mRNA was detected along a relatively large portion of the tubuli, whereas LF antigen was found mainly in the very distal regions of the same tubuli. This indicates that LF is released by large regions of the tubuli and possibly reabsorbed in the most distal parts renal tubules. Nonetheless, it is suggested that LF may support the immune system via the reduction of free iron in the urine (Abrink, Larsson, Gobl, Hellman, 2000).

The immunomodulatory function of lactoferrin is well known. It interacts with specific receptors of the target cells (either epithelial cells and cells of the immune system), and also thanks to its ability to bind to bacterial wall LPS (Na et al., 2004). Acting via two mechanisms of intracellular signal transduction, i.e. *nuclear factor kappa B* and MAP kinase, LF modulates differentiation, maturation, activation, migration, proliferation and function of immune cells, T and B cells, neutrophils, monocytes/macrophages and dendritic cells belonging to the antigen-presenting cells (APC) (Gahr, Speer, Damerau, Sawatzki, 1991; Krzyżowska, Świątek, Fijałkowska, Niemiałtowski, Schollenberger, 2009; Siqueiros-Cendón et al., 2014) (Figure 3).

Macrophages are antigen presenting cells and their role in innate immune response is based on inducing the phagocytosis of foreign particles and subsequently releasing pro-inflammatory mediators. They also participate in adaptive immune system by stimulating specific T cells after antigen presentation. *In vitro* studies have shown that both human and bovine macrophages contain surface receptors for LF (Birgens, Hansen, Karle, Kristensen, 1983; Roseanu, Chelu, Trif, Motaş, Brock, 2000; Siqueiros-Cendón et al., 2014). LF is also involved in the suppression of proinflammatory cytokines and induction of interferon α/β (IFN/β), and affects the ability of macrophages to present antigens to $CD4^+$ T cells in the adaptive resistance (Latorre, Puddu, Valenti, Gessani, 2010; Siqueiros-Cendón et al., 2014). The major cytokine produced by macrophages is IL-12, acting as a stimulator of INFα secretion by differentiated Th1 and T cells of the immunological memory (Gately et al., 1998). LF also enhances the expression of adhesion molecules on endothelial cells, resulting in leukocyte infiltration at the inflammation site (Kim, Lee, Park, Choi, Kim, 2012).

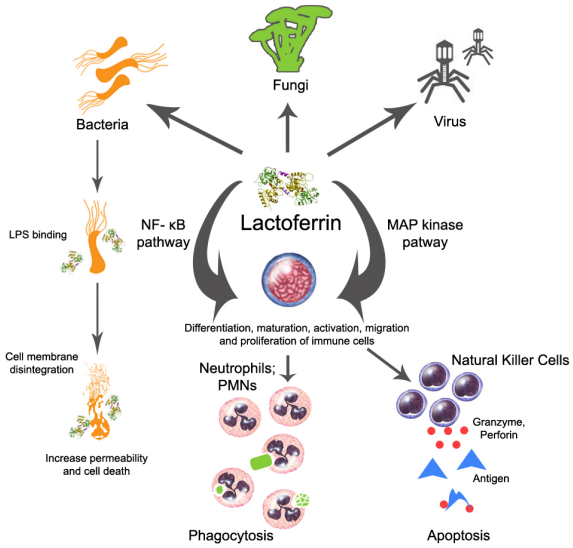

Figure 3. Role of lactoferrin in the activation of immune cells

Dendritic cells also exhibit phagocytic properties, participate in the differentiation of T cells, and regulate the functions of memory T cells (Siqueiros-Cendón et al., 2014). They also play an important role in the stimulation of

Th1 cells, resulting in cytokine secretion (Moser, Murphy, 2000). Similar to macrophages, dendritic cells are capable of binding hLF and bLF (Saidi et al., 2006). LF stimulates the function of dendritic cells, but also acts as alarming to promote the recruitment and activation of APCs and antigen-specific immune response (Yang, Rosa, Tewary, Oppenheim, 2009; Siqueiros-Cendón et al., 2014).

LF is involved in the regulation of B-cell functions and the subpopulation of T cells which express LF receptors (Legrand et al., 1997; Kawasaki, Sato, Shinmoto, Dosako, 2000). Oral administration of LF stimulates the secretion of IgG and IgA in the intestinal mucosa of mice (Sfeir, Dubarry, Boyaka, Rautureau, Tomé, 2004; Siqueiros-Cendón et al., 2014). LF reduces the inflammation response in allergic rhinitis, by inhibiting the activity of Th2, Th17 and regulatory T cells. It can promote Th1 responses, while inhibiting Th2 responses, and it causes T-cell receptor cross-linking, which leads to the inhibition of T-cell activation, reduces the release of inflammatory mediators, such as IL-5 and IL-17, and alleviates the degree of inflammation (Siqueiros-Cendón et al., 2014; Wang et al., 2013). Thanks to its ability to bind to cell surface receptors, LF has an ability to modulate the function of T cells and natural killer cells (Kanwar et al., 2015; Siqueiros-Cendón et al., 2014).

Conclusions

Lactoferrin, a multifunctional glycoprotein naturally occurring in mammals, influences a number of physiological and pathological processes. It participates in morphogenesis, iron homeostasis in the body, and thanks to its antimicrobial function and the ability to modulate the function of the cells of the immune system it can be described as the first line of defense in mammals. Therefore, researchers are trying to find the therapeutic application of LF in the prevention and/or treatment of cancer, bacterial diseases and inflammation. The use of LF is also recommended in dentistry, to reduce the risk of infections associated with dentures or implants.

Abbreviations

LF (Lactoferrin)
hLF (human lactoferrin)
rhLF (recombinant human lactoferrin)
gLF (goat lactoferrin)
cLF (camel lactoferrin)
bLF (bovine lactoferrin)
HBV (hepatitis B virus)
HCV (hepatitis C virus)
HIV (human immunodeficiency virus)
HPV (human papilloma virus)
HSV (herpes simplex virus)
HNSCC (head and neck squamous cell carcinoma)
Ig (immunoglobulin)
IL (interleukin)
INF (interferon)
LPS (lipopolysaccharide)
MAP kinase (mitogen-activated protein kinase)
TNF (tumor necrosis factor)

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