

Review

Antiinflammatory Activities of Lactoferrin

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Lactoferrin is a non-heme iron binding glycoprotein produced during lactation and by epithelial cells at mucosal surfaces. The protein is a prominent component of the first line of mammalian host defense and its expression is upregulated in response to inflammatory stimuli. In this paper, the antibacterial and immune modulatory properties of lactoferrin that contribute to host defense are reviewed. In addition, the results of recent preclinical and clinical studies demonstrating that lactoferrin acts as an inhibitor of dermal inflammatory cytokine production are summarized. The results indicate that lactoferrin may act as a potent anti-inflammatory protein at local sites of inflammation including the respiratory and gastrointestinal tracts.

Key teaching points:

- Lactoferrin is a multifunctional protein with antibacterial and immune modulatory activities and is a component of the first line of host defense.
- Lactoferrin can decrease dermal immune responses in both rodents and humans by inhibiting local production of cytokine mediators of inflammation.
- Lactoferrin also inhibits gut inflammation, most likely by similar mechanisms.
- Sufficient quantities of recombinant human lactoferrin have been produced for clinical testing of its efficacy in the treatment and prevention of gastrointestinal inflammatory conditions.

INTRODUCTION

This paper reviews the role of lactoferrin in mammalian host defense and, in particular, its mechanism of action with regard to regulation of immune responses in the first line of defense. Current information on the functions and mechanism of action of lactoferrin are reviewed. The production from filamentous fungi and testing of recombinant human lactoferrin for clinical studies and for nutritional and pharmaceutical use in humans are summarized. Also discussed are recent data obtained from preclinical and clinical studies that demonstrate the efficacy of lactoferrin as a potent anti-inflammatory agent in both dermal and gastrointestinal inflammatory conditions.

LACTOFERRIN: A MEMBER OF THE TRANSFERRIN FAMILY OF IRON BINDING GLYCOPROTEINS

Lactoferrin is an iron binding glycoprotein that consists of a single polypeptide chain of $M_r = 78\text{kD}$. It is the second most

abundant protein in human milk ($\sim 1\text{ g/L}$) [1,2] and is found in most exocrine secretions including tears, nasal secretions, saliva, intestinal mucus and genital secretions [3,4]. The protein also is expressed and secreted by the secondary granules of polymorphonuclear neutrophils [5]. The polypeptide structure of lactoferrin comprises two homologous domains that appear to have arisen by intragenic duplication [6]. The crystal structure of the protein has been resolved [7,8] and each domain binds one ferric and one carbonate anion. In addition, each domain contains one glycosylated site to which N-linked glycosidic residues are attached [9].

Lactoferrin is a member of the transferrin family of non-heme iron binding proteins [10]. The family includes transferrin, the major iron transporting protein in blood [11,12], the egg-white protein, ovotransferrin [13], the melanocyte protein, melanotransferrin [14] and a recently identified carbonic anhydrase inhibitor [15]. Members of the transferrin family are distinguished from other iron binding proteins by their unique anion requirement for binding of iron. The essential role of

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transferrin in regulating iron delivery to erythrocytes has been demonstrated by the characterization of Hpx mutant mice that carry a splicing defect in the transferrin gene resulting in very low levels of functional transferrin expression. These mice suffer from severe iron deficiency anemia, display a compensatory increase in intestinal iron absorption and develop tissue iron overload similar to patients with hemochromatosis [16]. With regard to its iron binding properties, lactoferrin differs from serum transferrin in its higher iron binding affinity and in its unique ability to retain iron over a broad pH range [17,18]. These differences, together with the differential tissue distribution of lactoferrin relative to other transferrins, contribute to the unique functional properties of lactoferrin.

Lactoferrin is a multifunctional protein to which several physiological functions have been ascribed (reviewed in [19, 20]). These include regulation of iron absorption in the intestine [21,22], promotion of intestinal cell growth [23], protection against microbial infection [24–26], regulation of myelopoiesis [26,27] and systemic immune responses [28,29]. This review focuses on the activities of lactoferrin that contribute to host defense.

LACTOFERRIN: A KEY COMPONENT OF THE FIRST LINE OF HOST DEFENSE

Although originally identified as an abundant protein in milk secretions, lactoferrin is expressed predominantly by surface epithelia and secreted into the mucosal environment. Thus, the protein is produced at high levels in nasal and tracheal passages and in gastric, genital and ophthalmic secretions. These secretions contain a variety of broad range antimicrobial and anti-inflammatory peptides and proteins that protect against chemical and microbial challenges. Finally, lactoferrin is also produced at high levels in neutrophils where it is stored in secondary granules and released during inflammation to contribute to their antimicrobial activity.

The expression of lactoferrin in the above regions responds rapidly and robustly to a variety of physiological and environmental challenges. The protein is strongly upregulated during airway [30,31] and gut inflammation [32–34] and in response to allergens [35] and other environmental insults including estrogens [36].

ANTIBACTERIAL PROPERTIES OF LACTOFERRIN

The antibacterial functions of lactoferrin have been substantiated by both *in vitro* [37–39] and *in vivo* [40,41] evidence. It appears that two different mechanisms involving two separate domains of the protein contribute to the antimicrobial functions of lactoferrin. The first mechanism is a bacteriostatic effect

related to the high iron binding affinity of the protein that deprives iron-requiring bacteria of this essential growth nutrient [37,42,43]. Since the bacteriostatic properties of lactoferrin are due to its iron binding ability, the protein is capable of retarding the growth of a broad range of microorganisms including a variety of gram negative and gram positive bacteria and certain yeasts [42,44]. However, bacteriostasis is often temporary because some gram negative bacteria adapt to iron restrictive conditions by synthesizing low molecular weight iron chelators (siderophores) that can remove iron from lactoferrin. The efficacy of bacteriostasis is dependent on the iron status of lactoferrin and is overcome by saturation of lactoferrin with iron [45]. In this regard, it should be noted that lactoferrin expressed in mothers' milk is only 5% to 8% saturated with iron and therefore would be expected to function as an efficient bacteriostatic agent.

The second antibacterial property of lactoferrin is due to a direct bactericidal function within the protein. Lactoferrin has a direct bactericidal effect against some gram negative and gram positive bacteria that cannot be attributed to simple iron deprivation. Apolactoferrin can cause a rapid loss of bacterial viability that cannot be reversed by the addition of exogenous iron to the growth medium [37,46]. At physiological concentrations, apolactoferrin directly damages the outer membrane of gram negative bacteria by causing the release of lipopolysaccharides (LPS) [47]. Recent results suggest that a cationic domain at the N-terminus of lactoferrin is responsible for its bactericidal properties. Synthetic peptides containing this cationic domain (amino acid residues 18–40) of human lactoferrin have a more potent bactericidal effect [48] and lead to a greater release of LPS [49] than intact lactoferrin [48]. The N-terminal amino acids responsible for the bactericidal effect are distinct from those involved in iron binding indicating that the bactericidal effect of lactoferrin is exerted by a mechanism distinct from metal chelation.

REGULATION OF IMMUNE RESPONSES BY LACTOFERRIN

A significant body of evidence has accumulated in recent years to support a role for lactoferrin in regulation of host immunity [28,29]. Lactoferrin is expressed in neutrophil secondary granules [5] and has been reported to have both positive [50] and negative [27,51–53] regulatory effects on myelopoiesis. Systemic infection with bacteria is accompanied by a rapid rise in serum levels of lactoferrin secreted from granulocytes [54] and a concomitant decrease in serum iron levels (hypoferremia). Studies have demonstrated that administration of lactoferrin can protect mice against a lethal dose of *E. coli* when administered either intraperitoneally (i.p.) or intravenously (i.v.) [41,55] prior to i.p. or i.v. infection with the bacteria. Both *in vitro* and *in vivo* studies suggest that this

prophylactic effect of lactoferrin involves an inhibition of production of several cytokines including tumor necrosis factor α (TNF- α) and interleukin-1 β (IL-1 β) that are key mediators of the inflammatory response leading to death from toxic shock [28,29]. It has been proposed that this inhibition of TNF- α release by lactoferrin is due its ability to act as an anti-endotoxin by binding to the lipid A moiety of LPS released from lysed bacteria thereby inhibiting subsequent binding of LPS to CD14 receptors on macrophages where it initiates a proinflammatory response [56]. However, the identification of receptors for lactoferrin on the surface of myeloblasts [57], monocytes [58], macrophages [59], and lymphocytes [60], in addition to epithelial cells involved in local production of TNF- α [20], suggests that lactoferrin may have a direct effect on regulation of cytokine production by these cells via receptor mediated signaling pathways.

LACTOFERRIN INHIBITS ALLERGEN INDUCED CUTANEOUS IMMUNITY

To test the hypothesis that lactoferrin may regulate local TNF- α dependent inflammatory responses in an LPS independent manner, we recently used a mouse model of allergen induced skin inflammation [61]. Previous studies indicate that solvent allergens act as potent stimulators of cutaneous immunity. Upon topical administration of a solvent allergen such as oxazolone, hapten-primed epidermal Langerhans cells (LC) undergo phenotypic changes and migrate from the epidermis via afferent lymphatics to the draining lymph nodes where they accumulate as immunostimulatory antigen presenting dendritic cells (DC) [62,63]. The response can be measured by the number of LC remaining in the epidermis and the number of DC accumulating in the draining lymph nodes before and after allergen treatment. The process is a critical event during the initial phase of skin sensitization and other forms of cutaneous immune response. The reason is that a proportion of the LC, which are stimulated to migrate from the skin, bear high levels of antigen and transport it in an immunogenic form to the lymph nodes where primary immune responses are provoked. Recently, it has been demonstrated that epidermal cytokines provide the molecular signals necessary for LC migration and DC accumulation in the lymph nodes. Effective mobilization of LC is dependent upon the availability of IL-1 β , a constitutively expressed and inducible product of LC, and TNF- α , an inducible product of keratinocytes (KC). Administration of allergen to the skin surface stimulates release of IL-1 β from epidermal Langerhan cells (LC), which in turn stimulates the synthesis of TNF- α by neighboring keratinocyte epithelial cells.

Previous experiments demonstrated that the effects of allergen on stimulation of LC migration and DC accumulation can be mimicked in mice if either TNF- α or IL-1 β is administered

intradermally in the absence of contact allergen [64–66]. Conversely, intravenous administration of antibodies to either cytokine is sufficient to inhibit LC migration and lymph node accumulation of DC. Thus, this is an extremely attractive model to determine the role of lactoferrin in modulating IL-1 β and TNF- α dependent local inflammatory responses initiated in an LPS independent system.

A potential role for lactoferrin in regulation of cutaneous immunity was originally suggested by the observations that a) lactoferrin is produced at high levels in human individuals with skin allergic reactions [35], b) the protein is produced locally in the epidermis of normal skin and c) lactoferrin exhibits competitive binding to putative receptors on keratinocyte cells, thereby indicating a potential to directly modulate the function of these epidermal cells [61,67].

To address the role of lactoferrin in regulating cutaneous inflammation, mice were sensitized by topical administration of oxazolone, a potent contact allergen and administered recombinant murine lactoferrin [68] by either intradermal injection or topical administration in aqueous cream. To determine whether topical administration of lactoferrin would deliver the protein to epidermal keratinocyte cells, exogenous radiolabeled lactoferrin was applied topically to mouse skin in aqueous cream and transverse sections were analyzed for uptake of the labeled protein. Autoradiographic analysis of these sections indicated that the protein appeared to be absorbed through the hair follicles and is concentrated in keratinocyte cells in the follicular regions of the epidermis.

Analysis of the effects of lactoferrin on oxazolone induced cutaneous immune response demonstrated that administration of the protein by either route resulted in a dose dependent inhibition of LC migration and accumulation of DC within the draining lymph nodes. Surprisingly, the inhibitory effect of lactoferrin was independent of its iron saturation status suggesting that its immune regulatory activity may be independent of its iron binding function. The inhibitory effect was also observed when the inflammatory response was initiated in the absence of allergen by IL-1 β but was not observed when TNF- α was used as the initiating stimulus. Taken together, these results indicate that lactoferrin functions downstream of IL-1 β by interacting directly with keratinocyte cells to down-regulate the de novo production of TNF- α . Further, the findings demonstrate that lactoferrin can directly inhibit local inflammatory responses in vivo by a mechanism independent of its ability to bind LPS.

PRODUCTION OF RECOMBINANT LACTOFERRIN FROM FILAMENTOUS FUNGI

In order to produce sufficient quantities of the human protein for clinical testing in humans, we previously reported the characterization of recombinant human lactoferrin produced in

the filamentous fungus, *Aspergillus awamori* [69,70]. The protein has been produced at commercial scale and purified to homogeneity. The purified protein is indistinguishable from natural human lactoferrin in terms of its three dimensional structure [71] and biological activities, including iron and receptor binding functions and antibacterial activity [69,72]. The protein has undergone safety and toxicity testing in animals and humans for both oral and topical administration and has shown no adverse indications at concentrations up to 5g/kg [72].

INFLUENCE OF LACTOFERRIN ON CUTANEOUS INFLAMMATION IN HUMANS

The preclinical studies summarized above predicted that topical administration of lactoferrin to humans may influence the development of cutaneous inflammatory reactions. In order to determine whether the inhibitory activity of lactoferrin on LC migration observed in the mouse also extended to human allergic skin reactions, a similar study was carried out in human volunteers using purified recombinant human lactoferrin [73]. In this experiment, human volunteers were treated by topical administration of the contact allergen, diphenylpicryl ether (DPC), in the presence or absence of topically applied recombinant human lactoferrin [73]. As previously observed in mice, analysis of punch biopsies taken from these volunteers demonstrated a significant depletion of epidermal LC induced by DPC that was markedly inhibited by administration of lactoferrin. The application of DPC to these individuals resulted in a local inflammatory reaction that was characterized by erythema and an infiltration of leukocytes into the skin, while the administration of lactoferrin was associated with a reduction in erythema and a decrease in the magnitude of the leukocyte response. Thus, the inhibition of allergen induced LC migration by lactoferrin is associated with a decrease in the severity of local cutaneous inflammatory reaction.

ANTIINFLAMMATORY ROLE OF LACTOFERRIN IN THE GASTROINTESTINAL TRACT

The observation that lactoferrin can inhibit local inflammation by inhibition of TNF- α mediated immune responses predicts that lactoferrin exerts a similar antiinflammatory role at local sites of immune defense where the protein is expressed (e.g., the gastrointestinal tract, lung, uterus, etc). In this regard, the allergen induced cutaneous inflammation model used in the dermal studies described above is mechanistically similar to that observed in Crohn's disease in humans [74,75], and mimicked by TNBS induced colitis in mice [76,77] both of which are mediated by Th1 cell dependent inflammatory responses. Consistent with the hypothesis that lactoferrin may play an

important role in modulation of gastric inflammation, the protein is expressed in the gastric mucosa of the stomach [78,79] and interacts with receptors localized on gastric intestinal epithelial cells. Further, the expression of lactoferrin is elevated in the feces of patients with inflammatory conditions including ulcerative colitis and Crohn's disease [32-34]. Several recent studies carried out in mice have shown that administration of lactoferrin can reduce gastritis induced by *Helicobacter felis* [80] and protect gut mucosal integrity during lipopolysaccharide-induced endotoxemia [81,82]. While results from human clinical trials to address the efficacy of the protein in regulation of gut inflammatory conditions have not yet been reported, the availability of recombinant human lactoferrin, together with the recently obtained positive results regarding the safety of orally delivered recombinant protein in phase I clinical trials [72,83], indicate that efficacy testing is indeed imminent.

CONCLUSION

Lactoferrin is a prominent component of the mucosal defense system whose expression is upregulated in response to inflammatory stimuli. The protein contributes to mammalian host defense by acting as both an antibacterial and anti-inflammatory agent. The anti-inflammatory activity occurs through inhibition of binding of lipopolysaccharide endotoxin to inflammatory cells, as well as through interaction with epithelial cells at local sites of inflammation to inhibit inflammatory cytokine production.

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