REGULAR REVIEW

Biological role of lactoferrin

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Lactoferrin is an iron-binding protein closely related in structure to the serum iron transport protein transferrin. Unlike transferrin, only traces are normally present in serum, and it is instead found mainly in milk and other external secretions, and in the secondary granules of neutrophils. Although lactoferrin was first isolated 30 years ago, its biological role remains unclear. Some aspects of its function were discussed about 12 years ago in this journal,[1] and this review will attempt to reassess the function of lactoferrin in the light of the large amount of new information that has accrued since then.

Knowledge of the structure of lactoferrin has been advanced by recent *x* ray crystallographic studies, and the structure and iron binding properties of lactoferrin are reviewed in detail elsewhere.[2 3] Briefly lactoferrin, like transferrin, reversibly binds two ferric ions, for which synergistic binding of an anion, usually bicarbonate or carbonate, is necessary. However, its affinity constant for iron is 300 times greater than that of transferrin, and even in the presence of a competing iron chelator such as citrate it can retain iron down to pH 3 or less while transferrin loses it at pH 5. Unlike transferrin, lactoferrin is strongly basic. Human lactoferrin has been cloned and sequenced[4] and the recombinant protein expressed in baby hamster kidney cells.[5]

Lactoferrin in milk

Human milk is particularly rich in lactoferrin, the concentration ranging from about 7 g/l in colostrum to about 1 g/l in mature milk, though it may rise again towards the end of lactation.[6 7] A similar pattern is seen in the cow, but importantly the concentration in mid-lactation milk is very much lower, only about 0·1 g/l.[8] Lactoferrin concentrations were normal in iron deficient mothers,[9] but lower in mothers who were generally malnourished,[10] which suggests that protein energy malnutrition rather than iron influences lactoferrin synthesis in the mammary gland. Although lactoferrin is found in the milk of some other species, it is completely absent in others, such as the rat, rabbit, and dog.[6] The milk of the rat and rabbit do however contain significant amounts of transferrin, but the milk of the dog contains neither protein, even though the iron content is exceptionally high.[6] The fact that lactoferrin is absent from the milk of some species is a point worth bearing in mind when considering its possible biological function.

Human milk contains 3·6–12·5 μmol/l of iron, and of this only 60–70% is in the whey fraction, the remainder being in the lipid fraction (11–20%) or bound to casein (2–14%).[11] As a consequence, milk lactoferrin is only 6–8% saturated with iron, presumably because of the difficulty in gaining access to iron in the lipid fraction or casein micelles.

Association of lactoferrin with other molecules

Lactoferrin is very prone to binding to other macromolecules. These include other milk proteins such as IgA, casein, secretory component, albumin, lysozyme, and β-lactoglobulin.[12] Lactoferrin also binds to DNA.[13] A report of multiple forms of lactoferrin in human milk, some of which exhibit ribonuclease activity,[14] may arise from an interaction between lactoferrin and milk ribonuclease. Bovine milk ribonuclease is, like lysozyme, a small basic protein (molecular weight 13 600),[15] and some ribonuclease activity in human milk was also associated with a molecule of this size.[14] So far no clear biological role has been established for this propensity of lactoferrin to interact with other proteins.

Biological functions of lactoferrin

BACTERIOSTATIC ACTIVITY

Almost all bacteria require iron for growth, and because of their iron sequestering properties, the iron free (apo) forms of lactoferrin and transferrin are able to impede iron utilisation by bacteria. A large number of studies, reviewed elsewhere,[16] have demonstrated a bacteriostatic effect and in some cases a bactericidal effect of lactoferrin in vitro on a wide range of microorganisms, including Gram positive and Gram negative bacteria, aerobes, anaerobes, and yeasts. However, mechanisms other than simple iron withholding may be involved in the antibacterial action of lactoferrin, such as blockade of microbial carbohydrate metabolism[17] or destabilisation of the bacterial cell wall, perhaps through binding of calcium and magnesium.[18] Lactoferrin may synergise with other antibacterial proteins such as lysozyme, which is also present in milk. In this case even the iron saturated form is active, and inhibition results from agglutination by lactoferrin of bacteria

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whose cell wall has been modified by lysozyme.[19] Antibodies can also enhance the bacteriostatic action of lactoferrin,[20] probably by blocking production or uptake of microbial siderophores.

Lactoferrin may also possess antiviral activity, as it could protect mice against polycythaemia due to Friend virus.[21] This appears to be an indirect effect, perhaps via a reduction in target cell proliferation, and is probably related to the proposed inhibitory role of lactoferrin in myelopoiesis (see below).

In vivo, lactoferrin in milk might exercise its inhibitory effect on microbial growth in the mammary gland, in the intestine of the newborn, or both. Its role as a defence against infection in the human mammary gland appears not to have been investigated, but in cattle concentrations of lactoferrin increase during intramammary infection,[22] suggesting a possible role as a mammary non-specific defence mechanism. Conditions in the bovine mammary gland immediately before parturition and during involution favour antimicrobial activity of lactoferrin as concentrations of bicarbonate are higher and those of citrate lower than in milk.[22 23] Thus lactoferrin might perform a role in preventing infection of the mammary gland, particularly at parturition and involution.

It has frequently been suggested that the antimicrobial activity of lactoferrin plays a part in the selection of the intestinal flora of the newborn and preventing colonisation by enteropathogenic organisms. The conditions in the intestine of the newborn may be more favourable for lactoferrin than those in the lactating mammary gland, as citrate is rapidly absorbed and intestinal fluid has a high concentration of bicarbonate, although this function might be adversely affected by the proteolytic enzymes present in the intestine, as discussed below. Nevertheless, despite the wealth of in vitro data, attempts to establish an in vivo antimicrobial role for lactoferrin have generally yielded disappointing results. The much quoted experimental study of Bullen *et al*,[20] which suggested a protective effect of lactoferrin in newborn guinea pigs infected with *Escherichia coli* is open to other interpretations, as discussed previously in this journal.[1] However, addition of chicken ovotransferrin to cows' milk was able to reduce mortality and bacterial counts in young guinea pigs infected with enteropathogenic *E coli*.[24] Two more recent clinical studies have analysed the faecal flora of infants fed bovine lactoferrin (in one case in conjunction with bovine IgG) and failed to find any significant difference between infants fed lactoferrin-supplemented formula and controls fed unsupplemented formula.[25 26] However, differences between bovine and human lactoferrins (such as resistance to proteolysis, discussed below) may mean that bovine lactoferrin is not an appropriate substitute for the human protein, and recombinant DNA technology now offers the possibility of obtaining sufficient human lactoferrin for clinical trials.

Thus although it is now well established that breast feeding offers protection to the newborn infant against gastrointestinal infection, the part played by lactoferrin must still be considered unproved until the wealth of in vitro data showing antimicrobial activity is supported by convincing in vivo data, particularly from clinical trials. The possibility that lactoferrin might exert a systemic protective effect also deserves further investigation, as studies in mice have shown that lactoferrin has a protective effect in experimental *E coli* septicaemia that is independent of its iron content.[27]

ROLE OF LACTOFERRIN IN INFLAMMATION AND THE IMMUNE SYSTEM

A number of studies suggest that lactoferrin may mediate some of the effects of inflammation and have a role in regulating various components of the immune system. It was proposed some years ago that lactoferrin released by degranulating neutrophils mediated the hyposideraemia of inflammation by removing iron from plasma transferrin and short circuiting it to macrophages of the reticuloendothelial system, where it was incorporated into ferritin.[28] However, iron uptake by macrophages from lactoferrin is at best extremely slow,[29] and there is no recycling of the protein as occurs with transferrin, as lactoferrin that has bound to monocytes cannot subsequently rebind to these cells.[30] In addition, the rate of exchange of iron between transferrin and lactoferrin at physiological pH is likely to be extremely slow, and finally neutrophil-derived lactoferrin differs from milk lactoferrin, which was used in most studies, in lacking the terminal fucose residues in its glycan chains that are required for binding to macrophages.[31] It has also been found in mice that interleukin-1 induces hypoferraemia even in the presence of neutropenia,[32] suggesting that lactoferrin is unimportant. Thus it now seems unlikely that lactoferrin plays a significant part in the hypoferraemia of inflammation.

Lactoferrin might also contribute to the bactericidal activity of neutrophils by two opposing mechanisms. In the apo form it may perform an iron withholding function and prevent growth of phagocytosed bacteria.[33] On the other hand iron-lactoferrin may provide iron that can catalyse the production of free radicals which lead to microbial killing within the phagolysosome of phagocytic cells.[34] The ability of lactoferrin to promote formation of free radicals is probably confined to acidic conditions such as those within the phagolysosome, as at physiological pH it is more likely that lactoferrin inhibits radical production by scavenging catalytic 'free' iron.[35] A role for lactoferrin in the antimicrobial activity of neutrophils is supported by the finding that patients whose neutrophils lack specific granules suffer from recurrent infections.[36]

Lactoferrin may also act as an inhibitor of secretion of granulocyte-monocyte-colony stimulating factor (GM-CSF).[37] This complex area has been intensively investigated and is reviewed fully elsewhere.[38] In summary, lactoferrin appears to act by decreasing synthesis of interleukin-1 which is necessary for GM-CSF production. Iron-lactoferrin was more effective than apo lactoferrin but it is not known what if any part is played by iron. Lactoferrin isolated

from neutrophils lacking receptors for the Fc region of immunoglobulin or from neutrophils of leukaemia patients was inactive, though the reason for this is unclear as possible differences between the active and inactive forms of lactoferrin were not investigated.

The role of lactoferrin as an inhibitor of myelopoiesis is somewhat controversial, however, as some reports have failed to find any inhibitory effect.[39 40] Some aspects of the original studies such as the extremely low concentrations of lactoferrin required for activity (10^{-17} M) and a discrepancy between the isoelectric point of the active component (6·5)[37] and purified lactoferrin (8·7)[41] require explanation. Further work is needed, particularly with a view to establishing how lactoferrin itself initiates these events at the molecular level, before its role in myelopoiesis can be regarded as fully established.

Lactoferrin has been reported to affect various aspects of the immune system, such as inhibiting in vitro antibody synthesis,[42] regulating monocyte/macrophage cytotoxic activity,[43 44] and affecting lymphocyte proliferation.[45–48] In most of these studies the mechanism by which lactoferrin carries out these functions was not established. The effect on lymphocyte proliferation is unclear, as some authors have reported enhancement[45] and others inhibition.[46 47] Possibly this may depend upon culture conditions, as recent work has suggested that apolactoferrin can overcome the inhibitory effect of 'free' iron on lymphocyte proliferation, but itself inhibits proliferation when iron is bound to transferrin.[48] Activation of T lymphocytes induces the presence of lactoferrin-binding molecules in the cell membrane,[45 49] and binding to B cells has also been reported.[50] However, further work is required before an immunoregulatory role for lactoferrin can be clearly established.

ROLE OF LACTOFERRIN IN THE ABSORPTION OF IRON

The greater bioavailability of iron and higher concentration of lactoferrin in human milk compared with cows' milk suggests that lactoferrin might promote iron absorption in breast fed infants. In a previous review in this journal it was suggested that lactoferrin actually had an opposite effect, and served as an additional control of iron absorption during the neonatal period.[1] More recent clinical trials have failed to demonstrate any improvement in iron absorption in infants fed formula milk supplemented with (bovine) lactoferrin.[51 52] A study in rats did show that feeding lactoferrin improved iron status[53] but the rat is a poor model for such studies as the milk of this species does not contain lactoferrin. Studies in piglets,[54] weanling mice,[55] or infant rhesus monkeys[56] showed that the bioavailability of iron bound to lactoferrin was no better than that of inorganic iron.

If lactoferrin were to have a role in iron absorption one might expect a receptor to exist in mucosal cells. Lactoferrin-binding proteins have been identified in the microvillus membranes of rabbit,[57] mouse,[58] and rhesus monkey enterocytes.[59] The molecular weights and binding characteristics of these proteins differ between species. However, there is so far no evidence that these membrane proteins actually mediate uptake or transport of lactoferrin-bound iron in mucosal cells. Given that there are also studies suggesting that lactoferrin may inhibit iron absorption, reviewed previously,[1] our earlier conclusion that lactoferrin controls rather than promotes iron absorption in the neonate still seems to be valid.

PROTEOLYTIC DEGRADATION OF LACTOFERRIN AND SURVIVAL IN THE GASTROINTESTINAL TRACT

A role for lactoferrin in iron absorption or establishment of the microbial flora of the gut presupposes a certain resistance to the conditions within the gastrointestinal tract. In vitro studies have found that apolactoferrin is more sensitive to the action of trypsin than iron-lactoferrin, although human apolactoferrin was less sensitive than bovine.[60] Lactoferrin is also degraded by gastric fluid from premature babies, though unlike transferrin or casein more degradation occurred at pH 3·2 than at pH 1·8.[61] However the gastric pH will be raised by the buffering capacity of milk proteins, and the intestinal flora and the velocity of transit also affect digestion of lactoferrin.[9] Studies of lactoferrin isolated from the faeces of newborn infants fed cows' milk supplemented with human lactoferrin or bovine lactoferrin showed that iron-lactoferrins survived better than the apo forms and, surprisingly, bovine lactoferrin was more resistant than human.[62] Partly digested proteins maintained their capacity to bind iron after excretion, and fragments of human lactoferrin obtained by acid hydrolysis were still able to bind to the putative rhesus monkey mucosal receptor, albeit with lower affinity than intact lactoferrin.[63]

Intact lactoferrin and fragments have been detected in the urine of premature infants receiving human milk,[64] arising apparently from lactoferrin absorbed across the gut.[13] This suggests that lactoferrin is partially degraded in the gut and that significant absorption of this protein occurs in the premature infant. However, some fragments may be present before ingestion, as in contrast to a previous report[65] we have recently found lactoferrin fragments in human and bovine milk.[66] It seems less likely that lactoferrin absorption occurs in term infants, as no difference in plasma lactoferrin concentrations was found between breast and bottle fed infants.[67]

ACTIVITY OF LACTOFERRIN AS A GROWTH FACTOR

Recent studies have suggested that lactoferrin may promote cell growth. As well as possibly promoting lymphocyte proliferation (see above), human lactoferrin also stimulated growth of various cell lines.[29 68] It also enhanced proliferation of rat mucosal crypt cells,[69] which suggests that it might play a part in maturation of the intestine in the newborn. The possibility that lactoferrin acts as a growth factor is supported by the presence of high concentrations of lactoferrin in some tumours,[70] although the

reasons for these high concentrations are unclear. In some cases growth promotion required iron[68] whereas in others iron was unimportant.[29 69] The mechanism by which lactoferrin is able to stimulate cell growth is therefore unclear, but seems unlikely to involve iron transport in an analogous manner to transferrin, as lactoferrin is not internalised and little or no iron is taken up from lactoferrin.[29] It might involve activation of membrane oxidoreductase activity.[71]

Conclusion: the biological function of lactoferrin—a unifying hypothesis

Over the past 10 years there has been considerable research on the function of lactoferrin, much of it in areas unrelated to the traditional fields of iron absorption and antimicrobial activity. Despite this, no clear biological role for lactoferrin has been established. Its antimicrobial activity, so well documented in vitro, still awaits convincing in vivo evidence. New studies of iron absorption have generally failed to show that iron in lactoferrin has a higher bioavailability than iron salts, and there is still no direct evidence that lactoferrin actually mediates uptake across the mucosa. Although lactoferrin-binding proteins have been identified in mucosal membranes, these could just as easily serve to regulate iron transport as enhance it. A role in hypoferraemia now seems unlikely, and effects on myelopoiesis and the immune system are either controversial or essentially phenomenological, with little information on how lactoferrin actually mediates these effects. Although lactoferrin receptors or binding proteins have been reported from a wide variety of cells and tissues the molecular structure and binding characteristics of these putative receptors varies considerably, and in many cases binding is relatively non-specific. It may well be that the various apparently unrelated effects of lactoferrin on the immune system arise from its ability to bind to macrophages and lymphocytes. This could alter the surface charge of the cell membrane, with consequent effects on the various cell-cell interactions that have a crucial role in many immunological mechanisms.

We believe that current evidence suggests that lactoferrin, despite its structural similarity to transferrin, does not act as an iron transport protein. Instead, we propose that its role is that of a specialised iron-scavenging protein, designed to act particularly under conditions where transferrin would be less effective at binding iron due to reduced pH, such as exist in the gastrointestinal tract or inflammatory lesions. By binding iron under these conditions it would render harmless 'free' iron that might otherwise cause free radical-mediated damage to sensitive tissues, reduce absorption of unwanted iron in the immediate postnatal period, and decrease its availability to micro-organisms. The ability of lactoferrin to bind to a wide variety of cells and tissues, perhaps without the need for expression of a truly specific receptor, would enhance its ability to prevent free radical-mediated damage or microbial invasion. It would also help to ensure its eventual removal and catabolism via the reticuloendothelial system or by biliary excretion. Further studies, particularly of lactoferrin-cell interactions, may help to establish the validity of this proposal.

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