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Mammalian lactoferrin receptors: structure and function

Y. A. Suzuki, V. Lopez and B. Lönnerdal*

Department of Nutrition, University of California, Davis, California 95616 (USA), e-mail: bllonnerdal@ucdavis.edu

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Abstract. Mammalian lactoferrin (Lf) receptors are suggested to have pivotal roles for mediating multiple functions of Lf. In this review, we focus on current knowledge of the structure and function of mammalian Lf receptors, mainly the first cloned Lf receptor that has been shown to be expressed in the infant small intestine at high levels but also in virtually all other tissues. The small intestinal Lf receptor takes up iron from Lf into cells and presumably exerts other physiological functions. Other Lf receptors in various tissues have also been reported to mediate some functions of Lf, such as modulating immune function, inhibiting platelet aggregation and enhancing collagen gel contractile strength. The detailed mechanisms behind the receptor-Lf interactions still need to be elucidated.

Key words. Lactoferrin, lactoferrin receptor, mammalian receptor, iron metabolism, immune function.

Introduction

The high concentration of lactoferrin (Lf) in human milk and its capacity to bind a major part of breast milk iron suggested that Lf may have a physiological function in the newborn infant. This hypothesis was supported by in vitro studies, which showed that Lf resists proteolytic digestion, particularly when the pH for pepsin digestion is not very low (as in infants) and when it is fully iron-saturated [1, 2]. Suggested functions of Lf in the gastrointestinal tract include facilitation of iron absorption, modulation of mucosal immunity and stimulation of mucosal differentiation [3]. Lf is found in a variety of tissues and is specifically recognized by cellular components also present in many tissues. Multiple biological activities of Lf depend on its target cells and on the presence of specific receptors (LfRs) at their surfaces. Research on LfRs has usually been initiated by investigations on binding kinetics of Lf to target cells. According to such kinetic work, different tissues or cell types appear to express their own LfRs, and their characteristics vary among different cell types [4]. A difficult part of binding experiments with Lf is that there is always a risk of picking up 'pseudo-receptors' since Lf has high pI and, thus, a net positive charge in most binding assays. It is therefore necessary to perform binding studies on Lf with caution.

We have investigated small intestinal LfRs in various species and purified a small intestinal human LfR (SI-hLfR) from infants. The complementary DNA (cDNA) of the SI-hLfR was cloned, and some biochemical characteristics and its tissue distribution have been revealed [5]. Here we comprehensively review current knowledge about the SI-LfRs, including their suggested functions. Among the many studies on mammalian LfRs characterized in numerous cell types, two types of liver LfR have been identified as LDL receptor related protein (LRP) and as asialoglycoprotein (ASGP) receptor. Their modes of function are also discussed. In addition, current knowledge of a lymphocyte LfR (LC-LfR), a monocyte LfR (MC-LfR), and other LfRs will be sorted out (table 1), which will hopefully help to clarify the picture of LfR research and provide guidelines for what can be expected in future work.

Intestinal LfRs

Implications of the existence of intestinal LfRs

In 1979, the presence of LfRs in the small intestine was suggested for the first time by Cox et al. [6]. They incubated biopsies from adult human small intestine with hLf,

* Corresponding author.

Table 1. Summary of characteristics of mammalian lactoferrin receptors

Tissue	Species	Ligan	Molecular d weight	Glyca	Other n name	Suggested function	Reference
Small intestine	human (infant)	hLf	37 kD	4 kD	intelectin		[5]
Small intestine	human (Caco-2)	hLf	34 kD		intelectin	iron uptake	[5]
Small intestine	mouse	mLf	34 kD		intelectin		[32]
Small intestine	mouse	hLf	130 kD	25 kD			[23]
Small intestine	rabbit	hLf	105 kD				[14]
Liver	rat	hLf	600 kD		LRP	inflammation	[66]
Liver	rat	bLf	45 kD		ASGPR		[82, 83]
Monocyte	human (THP-1)	hLf	50, 60, 80 kDa			inflammatory response	[114]
Monocyte	human	hLf	55 kD		CD14	inflammatory response	[109]
Lymphocyte	human	hLf	100, 110 kDa			maturation of lymphocyte	[84]
Lymphocyte	human (Jurkat)	hLf	105 kD			maturation of lymphocyte	[88]
Platelet	human	hLf	105 kD			inhibition of platelet	[118]
Fibroblast	human	bLf	600 kD		LRP	collagen gel contractile	[73]
Bone	rat and human	bLf	600 kD		LRP	mitogenesis of osteoblasts	[76]
Brain	bovine	bLf	600 kD		LRP	Lf transport	[72]

serum transferrin (Tf), and ovo-Tf, and found that only hLf, but neither serum Tf nor ovo-Tf, can deliver iron to human duodenal mucosa. In order for the small intestinal LfR to function, survival of Lf through the gastrointestinal tract without proteolytic degradation has been considered a requirement. Lf is present in human milk in considerable quantities: ~10 g/l in the colostrum and 1–2 g/l in mature milk. Therefore, it sounds reasonable to hypothesize that hLf is important for infants, especially during the early stages after birth. Fecal excretion of hLf has been assessed in full-term exclusively breast-fed infants [7]. Intact fecal Lf was detectable from the first week of life to the end of study at 22 weeks of age. The milder conditions of the neonatal gastrointestinal system, such as relatively high pH and low enzyme secretion, may enhance the persistence of Lf. It is therefore plausible, especially for infants, that Lf survives digestion in vivo and interacts with its specific receptor in the small intestine. In addition, the stability of orally administered bovine Lf (bLf) has been documented. The iron binding capacity of bLf was retained in feces of human infants after ingestion of bLf [8]. In adults, more than 60% of administered bLf was found to survive in the stomach [9], but recombinant hLf from transgenic cows was completely digested [10]. Thus, in human adults, bLf appears to be more resistant than hLf against proteolytic degradation, suggesting that hLf is biologically expected to be ingested orally only during infancy.

A lack of human intestinal material from infants directed the attention to different animal models. Rabbits, dogs and rats have been reported to have very little or no Lf (<50 μg/ml) in their milk, but primates, pigs and mice have significant concentrations of milk Lf (>200 μg/ml), whereas cows and goats have intermediate levels (<200 μg/ml) in their milk [11]. Therefore, primates, pigs and mice were considered possibly to be good models for investigations of small intestinal LfRs (SI-LfRs). Based on these facts and considerations, several kinetic studies have been conducted to characterize the SI-LfRs.

Binding studies of SI-LfRs

SI-LfRs have been reported in various species, and their kinetic characteristics were assessed in binding studies. A summary of the binding of Lf to various tissues in different species is shown in table 2. It should be noted that Lf has a high pI, and thus Lf is likely to have a positive charge in most binding assays. Taking this characteristic into consideration, receptors that can discriminate among Lfs of different species are likely to be more specific than those that are shown to bind Lf but not Tf. Note, however, that both hLf and bLf can bind specifically to rat brush border membrane vesicles (BBMVs), although rat milk contains no Lf [12]. It is therefore difficult to evaluate the biological significance of experiments with heterologous Lf. This is illustrated by the fact that brush border membranes from rats were found to bind bLf; however, bLf bound to rat Tf receptors (TfRs) [12]. This observation may explain the positive effect of bLf on iron absorption that has been observed in rats [13], but it is less likely that these findings are involved in the 'true' biological mechanisms of Lf.

It has been shown that hLf binds to brush border membranes from rabbit small intestine [14]. Specific binding of hLf occurred with an apparent dissociation constant

Table 2. Binding characteristics of Lf to small intestinal LfRs

Species	Ligand	Ineffective competitiors	Optimum pH	Binding sites (#/mg protein)	Dissociation constant	Ca^{2+} dependency	Reference
human	hLf	bLf	6.5–7.5	4.3×10^{14}	1 μM	partially	[5, 31]
macaque	macaque Lf, hLf	bLf, hTf	7	1.8×10^{15}	9 μM	ND	[16]
mouse	mLf, hLf, bLf	lysozyme, Fuc-BSA	5.5–6.5	5.3×10^{16}	3.5 μM	dependent	[22]
piglet	pLf	hLf, bLf, pTf	7	1.5×10^{15}	2.4–3.1 μM	ND	[25]
rabbit	hLf	Tf, fetuin, ovalbumin	ND	1.2×10^{13}	1 μM	dependent	[14]
bovine	bLf	bTf	7.5	$2–8 \times 10^{14}$	2.6–3.7 μM	ND	[27]

ND: not determined

(K_d) of ~1 μM. No significant inhibition was observed for serum Tf, calf fetuin or hen ovalbumin, but EGTA markedly inhibited binding of hLf, suggesting that divalent cations are required for the binding activity. Addition of Ca^{2+} to the incubation medium enhanced binding of hLf to the LfR. On the other hand, no specific binding of rabbit serum Tf was observed. In this study, Lf was of human origin, and it is uncertain to what degree human and rabbit Lf are similar.

The newborn macaque has been used as a model for human infants. First, macaque Lf was purified from milk, and its biochemical properties were confirmed to be very close to those of hLf, i.e. polyclonal anti-hLf antibodies cross-react with macaque Lf, but not with bLf [15]. The carbohydrate moiety of macaque Lf was shown to contain monosaccharides in similar proportions to those reported for hLf. These results suggested that the newborn macaque is a promising model for the study of the biological role of Lf. Binding studies have been conducted with BBMVs from biopsy samples from juvenile macaques [16]. The binding of macaque Lf to BBMV was time-dependent and saturable. Competitive binding experiments with excess unlabeled Lf showed that the binding was specific. Macaque Lf and hLf at 50-fold excess effectively inhibited binding of macaque Lf, while a similar excess of bLf and human Tf did not. These results provided evidence for the existence of a SI-LfR in infant macaque. Similar kinetic studies in BBMVs from fetal, adolescent and adult macaque intestine showed the presence of macaque SI-LfR throughout the life cycle. While the K_d was similar at all stages of development, the number of binding sites was highest during infancy, suggesting a physiological importance for macaque SI-LfRs during this age. Subsequently, binding of different variants of Lf to macaque SI-LfRs was explored. Although it has been shown that hLf is able to survive proteolytic digestion during passage through the gastrointestinal tract in newborn infants and that hLf was found in immunologically intact form in the stool of breast-fed infants [7], it is also true that some digestion of Lf will occur [17]. Structurally, Lf has two similar lobes that are connected via an extended helical loop, which is the most sensitive part for protease cleavage. Therefore, a proteolytic fragment of hLf was isolated and the binding characteristics to macaque BBMVs were explored [18]. The Lf fragment bound to macaque SI-LfR in a saturable manner and with a K_d similar to that of intact hLf, but competitive binding experiments indicated that intact hLf competed more effectively for binding than the fragment. It was also investigated whether degree of iron saturation of hLf affected binding to the macaque SI-LfR, since only 1–4% of hLf in human milk is iron-saturated [19]. Apo-hLf, partially saturated hLf and holo-hLf were all able to bind to the macaque SI-LfR, but apo-hLf was less effective to displace labeled hLf, suggesting that holo-hLf has a higher priority for interacting with the macaque SI-LfR. The macaque SI-LfR binding of manganese-hLf was also tested because hLf in human milk is known to bind manganese [20]. Manganese-hLf bound to macaque SI-LfR with an apparent K_d similar to that of iron-hLf. It should be noted that hLf does not bind to zinc under physiological conditions, but may only bind zinc weakly at extremely low ionic strength under in vitro conditions [21].

The mouse is the most convenient animal model, and genetic modifications are also possible. However, it should be noted that mouse milk contains both Lf and Tf. In contrast, human milk exclusively contains Lf but not Tf. Therefore, it should be kept in mind that the primary function of mouse Lf (mLf) may be quite different from that of hLf. Nevertheless, a mouse SI-LfR has also been described. Mouse BBMVs bound both hLf and bLf, although the affinity was lower than that for mLf [22]. Thus, the specificity of the binding is not as high as that of macaque SI-LfR. The pH optimum for binding of mLf to mouse SI-LfR (SI-mLfR) was found to be 5.5, and binding was Ca^{2+}-dependent. Both apo- and holo-Lf bound to the SI-mLfR to a similar extent. Lysozyme,

which is a highly cationic protein, did not inhibit binding of mLf, suggesting that the binding force is not a non-specific ionic attraction. Fucosylated bovine serum albumin did not inhibit the binding either, suggesting that fucose is not involved in the binding [23]. The existence of SI-mLfR may explain earlier findings of high iron absorption from Lf in mice [24].

The binding of hLf to human infant BBMVs was saturable at protein concentrations from 0.2 to 16 μM, whereas the ability of bLf to bind was around one-fifth of that of hLf. Neither bLf nor hTf competed with hLf for binding. Therefore, human SI-LfR (SI-hLfR) is much more specific to hLf than bLf, suggesting that this interaction does not fully depend on the positive charge of Lf, but more on a specific structure of hLf. The pH optimum for binding of hLf to the SI-hLfR was found to be 6.5–7.5, which would be consistent with the pH of the small intestine in infants. The apparent K_d was found to be ~1 μM. Complete deglycosylation of hLf by peptide-N-glycosidase F did not affect the binding property of hLf.

Piglets were also suggested to be an appropriate alternative model for investigating SI-LfRs. First, pig milk contains exclusively Lf (pLf), but not Tf (pTf). Second, piglets are less expensive than monkeys, and small intestine is often available without severe restrictions as for human or monkey tissues. BBMVs from piglet intestine were examined for pig LfR (pLfR) expression. The pLfR was found to be present throughout the intestine, and the number of binding sites and the dissociation constant were constant from birth until weaning. The pLfR did not bind hLf, bLf or pTf [25]. Isolated enterocytes from nursing piglet were subsequently shown to take up pLf [26]. These results suggest that the piglet is a useful model to study functions of the intestinal LfR.

Binding of bLf to bovine intestine has been reported. Brush border membranes from duodenum, jejunum, ileum, colon and epithelium overlying Peyer's patches have been found to bind to bLf with a K_d range of 2.6–3.7 μM. The optimum pH for bLf binding was between 6.0 and 7.5. However, bTf effectively inhibited the bLf binding, and reduced bLf binding down to 50% in colon and to 20% in the epithelium overlying the Peyer's patches of jejunum-ileum [27]. The specificity of the binding of bLf was not examined, and it cannot be excluded that most of the bLf binding observed was an interaction with the Tf receptor instead of a specific interaction with the SI-LfR. Binding studies with Lf from other species will give us a better idea with regard to the specificity of the remaining binding.

Cell culture systems have also been applied in this area of research. The HT-29 cell line has been utilized most extensively for binding and transport studies of Lf as a model for human small intestine; however, they are carcinoma cells and not infant derived. Binding of hLf to the HT-29-18-C1 cell line, one of the derivative cell lines from HT-29, was examined [28]. The apparent K_d was 60 nM for high-affinity binding sites and 700 nM for low-affinity binding sites. The iron saturation level did not seem to affect the binding significantly. It has been found that binding of bLf and hLf to both types of binding sites present on HT-29 cells was comparable, while mLf did not bind to the high-affinity site but bound to the low-affinity site with a K_d of 2.2 μM. Removal of Arg^2 of hLf had no effect on the binding, while additional removal of Arg^3 and Arg^4 abrogated hLf binding to the high-affinity binding sites. Removal of the additional Arg^5 resulted in no binding to either high-affinity or low-affinity sites, suggesting that Arg^5 is essential for binding to the low-affinity sites. Competition with heparan sulfate and chondroitin sulphate glycosaminoglycans inhibited hLf binding by 75 and 52%, respectively, suggesting that glycosaminoglycans may constitute the majority of hLf binding sites on HT29-18-C1 cells. The binding to cell membrane proteoglycans is not specific for Lfs. For example, fibroblast growth factor was sequestered by proteoglycans [29], which allows its presentation to a specific receptor and facilitates its interaction with this receptor [30]. It is therefore possible that the proteoglycans act as co-receptors and facilitate the contact between Lf and its functional high-affinity receptor.

Biochemical and molecular characteristics of the intestinal LfR (intelectin)

It has been challenging to isolate significant quantities of the SI-LfRs, which would allow detailed biochemical and structural analyses. The SI-LfRs were purified from solubilized brush-border membranes by affinity chromatography using immobilized iron-saturated hLf [23, 31]. The first SI-LfR purified was from the mouse [23]. It is notable for the purification of the mouse SI-LfR (SI-mLfR) that intestines from 1000 mice yielded about 110 μg of SI-mLfR and that recovery was only 1.2%. The SI-mLfR has a molecular weight of ~130 kDa and consists of a single polypeptide chain because no subunits were observed in SDS-polyacrylamide gel electrophoresis (PAGE) gel under reducing conditions. Isoelectric focusing indicated that the pI of the SI-mLfR was at pH 5.8. When digested by N-glycanase, which is known to split all N-linked oligosaccharides, including bi-, tri- and tetra-antennary glycans, whether fucosylated or not, the size of the SI-mLfR decreased in apparent molecular mass by 25 kDa. Digestion by N-acetyl-β-D-glucosaminidase B, cleaving mono-asialo, asialo-biantennary and asialo-triantennary glycans, resulted in reduction of the molecular weight by 25 kDa, while endo-N-acetyl-β-D-glucosaminidase H, digesting all oligomannosidic-type glycans, was ineffective. These results suggested that SI-mLfR is glycosylated by bi- and tri-antennary N-acetyllactosaminic-type glycans with a combined molecular weight of ~25

kDa. The molecular weight of deglycosylated SI-mLfR is thus estimated to be ~105 kDa.

We have recently documented the cloning of the cDNA encoding the functional SI-mLfR that was reported earlier as mouse intelectin [32]. It was first hypothesized that mouse intelectin in the small intestine mediates functions of mLf based on the homology with the cDNA sequence of the SI-hLfR. The cDNA for mouse intelectin was thus cloned by polymerase chain reaction (PCR). However, its molecular weight contradicts what has been reported earlier. This cDNA consists of only 313 amino acids, and the theoretical molecular weight is 34 kDa. In order to observe a direct interaction between SI-mLfR and mLf, both recombinant SI-mLfR (rSI-mLfR) and recombinant mLf (rmLf) were expressed in a baculovirus-insect cell expression system, and rmLf was purified by combination of cation exchange and Con A columns. The rSI-mLfR was then purified by affinity chromatography using immobilized rmLf. SDS-PAGE and Western blot of the eluted fraction revealed a single band of 102 kDa under reducing conditions in SDS-PAGE. The antibody for Western blot was raised against a synthetic peptide of the SI-LfR, which strongly suggested that the 102 kDa protein is the protein that we have expressed. The size of the purified protein appears to correspond to a trimer of the 34-kDa protein, but it did not dissociate into monomers by the reducing agent we used. The size of the rSI-mLfR that is observed by SDS-PAGE and Western blot is very close to that of the native SI-mLfR (nSI-mLfR) purified by Mazurier et al. Therefore, the nSI-mLfR may be the same protein as the rSI-mLfR, although it is necessary to characterize the rSI-mLfR in more detail.

Human SI-LfR (SI-hLfR) was found to have a molecular weight of 114 kDa [31], and it consists of subunits with a molecular weight of 38 kDa. Deglycosylation of the SI-hLfR with peptide N-glycosidase F, which is known to cleave all N-linked oligosaccharides, resulted in a decrease in apparent molecular mass by 4 kDa. Although SI-mLfR has been reported to consist of a single polypeptide [23], the SI-hLfR appeared to form subunits after reduction with β-mercaptoethanol, suggesting a disulfide bridge between subunits. The number of subunits per SI-hLfR molecule is three when simple mathematics is applied. The macaque SI-LfR was also isolated from infant macaque small intestine using the hLf affinity column. According to previous observations [18], hLf binds to the macaque SI-LfR, which we were able to confirm. The amino acid composition of the purified SI-hLfR and macaque SI-LfR is very similar. The amino acid sequence of SI-hLfR was only determined at the N-terminal end, and thus there was not enough information for cloning of its gene.

It took a decade to finally clone the cDNA of the SI-hLfR. Two amino acid sequences were determined from the purified native SI-hLfR (nSI-hLfR), and homologous sequences were found in both the Swall protein and human expressed sequence tag (EST) databases [5]. A putative cDNA for SI-hLfR was constructed according to those homologous ESTs, which revealed that the theoretical amino acid composition is close to the analysis of purified nSI-hLfR. PCR primers were designed for obtaining the entire coding region of the SI-hLfR based on the putative cDNA sequence of the SI-hLfR, and PCR cloning was performed on a cDNA library from human fetal small intestine. The deduced amino acid sequence of the SI-hLfR cDNA is homologous (81% identity) to the mouse intestinal lectin-like protein, intelectin [33], that is now characterized as the SI-mLfR. Cortical granule lectin (CGL) in *Xenopus* oocytes is also homologous with 67% identity [34]. Human intelectin has recently been characterized as a novel soluble lectin that recognizes galactofuranose in carbohydrate chains of bacterial cells [35]. The gene for this protein is identical to the SI-hLfR. Recombinant human intelectin has been reported to have affinity towards *d*-pentoses and *d*-galactofuranosyl residues in the presence of Ca^{2+}.

Whether the SI-hLfR cDNA encodes a functional receptor has been investigated by expressing the recombinant SI-hLfR (rSI-hLfR) in a baculovirus-insect cell system [5]. The rSI-hLfR was purified from the cell culture supernatant by immobilized hLf affinity chromatography in the presence of Ca^{2+}. Chelating Ca^{2+} by EGTA and lowering the pH caused release of rSI-hLfR from hLf, supporting that Ca^{2+} is at least partially required for the interaction between the rSI-hLfR and hLf. By using purified proteins attached to the ELISA plate, the interaction between hLf and rSI-hLfR was directly confirmed. ^{125}I-hLf bound to the rSI-hLfR with an apparent K_d of 360 ± 50 nM, indicating that the rSI-hLfR retained the capacity to bind hLf. There are small differences in biochemical characteristics between native and recombinant SI-hLfR. SDS-PAGE under non-reducing conditions revealed that rSI-hLfR tended to form tetramers, whereas native SI-hLfR appeared to form trimers. The apparent molecular mass was 136 kDa under non-reducing and 34 kDa under reducing conditions. One remaining issue is that although SI-hLfR was purified from BBMV, rSI-hLfR was in the cell culture supernatant.

A glycophosphatidylinositol (GPI)-anchored homologue to CGL was purified from *Xenopus* blastulas [36]. We thus found that the native SI-hLfR may be GPI-anchored. GPI-anchored proteins on the surface of Caco-2 cells were cleaved by phosphatidylinositol-specific phospholipase C (PI-PLC), and were then analyzed by both Western blot and eluted peak height from the immobilized hLf affinity column [5]. Relative band intensity of the PI-PLC treated sample was 4.6 times higher than that of control, and the peak height was 2.8 times higher for the PI-PLC treated sample, indicating that the SI-hLfR is GPI-anchored. This observation could explain how the SI-LfR

is associated with the cell surface. It should be noted that GPI-anchored proteins in general have been shown to have very diverse functions, such as transmembrane signaling [37] and endocytosis [38]. What the primary function of the GPI-anchored SI-hLfR is still remains to be elucidated, as discussed in the next section.

Suggested function of the SI-LfR

Facilitating iron absorption, especially in human infants, is a suggested function for the SI-hLfR (fig. 1A). Iron in mature human milk ranges from 0.2 to 0.4 mg/l, which is much lower than in infant formula in which the iron level is around 8–12 mg/l to prevent risk for iron deficiency. However, a majority of infants who are exclusively breast-fed are able to maintain their iron status at the same level as infants fed iron-fortified formula [39]. The reason for this high bioavailability of iron in human milk has been a long-time paradox. It has still not been fully elucidated, but the fact that almost all iron in human milk whey is bound to hLf strongly suggests involvement of hLf in the mechanism of high iron bioavailability from human milk.

The biological role of the intestinal SI-LfR-mediated mechanism has not yet been fully elucidated, but some experiments support its involvement in iron transport, which remains controversial. Translocation of hLf and hTf was studied in HT29-D4 cells [40]. Human Tf entered the cell by classical receptor-mediated endocytosis, whereas hLf and its receptor were not internalized. Nonpolarized monolayers were used for this experiment, and it is not certain that hTf entered from the basolateral side. Our recent study with polarized monolayers of Caco-2 cells, another human small intestinal like cell line, demonstrated that holo-hLf was internalized from the apical side but not from the basolateral side, whereas holo-hTf was internalized only from the basolateral side [41]. Transepithelial transport of hLf was also studied in another intestinal cell line, HT29cl.19A [42], and hLf was found to be transported across intestinal epithelial cells through two functional pathways. One is a major degradative pathway, in which ~90% of hLf was degraded and iron was released from hLf. The other is a minor pathway, transporting ~10% of hLf, that allows the passage of immunoreactive hLf and its bound iron. Intracellular iron depletion was found to upregulate expression of the intestinal SI-LfR, as suggested by increased binding of hLf to the cells, and the enhanced hLf binding resulted in an increase of ~30% in the uptake of hf-bound iron [43]. These results suggest that the intestinal SI-LfR is able to facilitate intestinal iron absorption dependent on body iron stores.

The small intestinal SI-hLfR cDNA was stably transfected into Caco-2 cells, to investigate receptor-mediated functions of hLf [5]. SI-LfR protein expression in

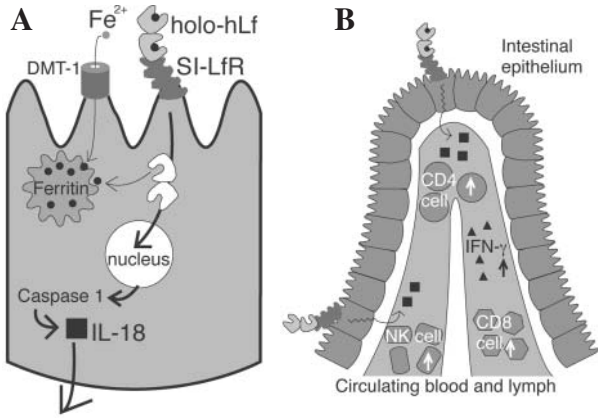

Figure 1. Suggested function of the SI-LfR. (A) The SI-LfR may facilitate iron absorption. Iron is usually transported through the apical membrane of the small intestine by DMT-1, a transporter for ferrous iron. Lf-bound iron cannot go through DMT-1 but may get inside the cell via the SI-LfR-mediated pathway. In the enterocyte, iron is stored in the iron storage protein, ferritin. The SI-LfR cannot take up Lf-bound iron from the basolateral side. Once getting inside the cell, Lf is targeted to the nucleus, where it is likely to act as a transcriptional factor to enhance biosynthesis of some signal proteins such as caspase 1 and IL-18, which then go into the circulation as a systemic signal (B). Orally administered Lf has been shown to enhance mucosal CD4, CD8 and NK cells, which is likely to be mediated by the SI-LfR.

SI-LfR-transfected cells was 3.2 times higher than that in mock-transfected cells, and binding of ^{125}I-hLf to SI-LfR-transfected cells was 74% higher than that to mock-transfected cells. When transfected cells were cultured on Transwell inserts and uptake and transport of ^{59}Fe-hLf within 3 h were examined, ^{59}Fe uptake from hLf into SI-LfR-transfected cells was significantly higher than that into mock-transfected cells and also higher than ^{59}Fe uptake by itself into LfR-transfected cells. On the other hand, the amount of ^{59}Fe transported into the basolateral side within 3 h was not significantly different. These results clearly showed that the small intestinal SI-LfR is able to mediate iron absorption from hLf for apical transport but not for basolateral transport. Although in vitro studies have indicated involvement of the SI-hLfR in iron absorption at the apical membrane, in vivo evidence is still needed. Some studies indicate no involvement of hLf in enhancing iron absorption, and thus the primary function of SI-hLfR is still debatable.

Another suggested mechanism for orally administered Lf in small intestine is enhancement of the immune system (fig. 1B). Oral administration of bLf has been shown to significantly decrease lung metastatic colonies in colon carcinoma-bearing mice [44]. One important factor in the anti-metastatic action was suggested to be enhanced natural killer (NK) activity [45]. In fact, $CD4^+$ and $CD8^+$ T cells and NK cells in the lamina propria of the small intestine were found to be increased after

oral administration of bLf or of its pepsin hydrolysate, bovine lactoferricin (bLfcin) [46]. These T cells and NK cells were also increased in blood and lymphoid tissues after bLf treatment [44]. Therefore, it is plausible that tumor cells may be killed by an increased number of T and NK cells. Thus, orally administered bLf and bLfcin may interact with SI-LfRs to enhance immunological activity and to inhibit metastasis. It has been reported that bLf stimulated synthesis of the pro-inflammatory cytokine interleukin-18 (IL-18) in small intestinal epitelium [46, 47]. IL-18 is known to be expressed by various cells, including macrophages, keratinocytes and intestinal epithelial cells [48], and to influence expression of a number of genes, such as interferon-γ (IFN-γ) in T and NK cells, as well as tumor necrosis factor α (TNF-α), IL-1β, and several other cytokines in cultured human blood cells [49]. It has also been shown that IL-18 enhances Th1 type T and NK cell responses and generates $CD8^+$ T cells [50], and also inhibits angiogenesis [51]. Recently, orally administered bLf at 300 mg/kg/day for 7 consecutive days was reported to induce caspase-1 and IL-18 in mouse intestinal mucosa [52]. Murine IL-18 is synthesized as pro-IL-18 (with a molecular weight of 24 kDa), and pro-IL-18 was induced by bLf and bLfcin but not by bTf in the small intestine. Pro-IL-18 is cleaved by caspase-1 into its active form with a molecular weight of 18 kDa [53]. A caspase-1 inhibitor has been shown to significantly decrease bLf-mediated induction of IL-18 in vitro. Although there is currently no connection between these immunological effects by bLf in mice and the SI-mLfR, it is conceivable that bLf interacts with SI-mLfR through the bLfcin structure, which enhances immunological activities by transducing some signals to synthesize IL-18 and caspase-1. There are several other lines of evidence from animal experiments that oral administration of bLf may enhance immune function through the SI-LfR-mediated mechanism. Systemic effects of orally administered Lf are more likely to be mediated via SI-LfRs. For example, bLf feeding augmented peritoneal macrophage activities in mice that were injected intraperitoneally with inactivated *Candida albicans*. Enhancement of macrophage activities was detected by nitric oxide production, inhibition of *Candida* growth, and IFN-γ and IL-12 levels in the peritoneal cavity [54]. In guinea pigs that were immunized with inactivated *Trichophyton mentagrophytes*, bLf feeding enhanced splenocyte secretion of a humoral factor that promotes fungicidal activity of macrophages [55]. Recently, clinical trials were conducted that explored immuno-modulatory effects of bLf. For instance, an effect of oral ingestion of bLf on skin fungal infection has been reported. The effect was not very potent, but dermatological symptom scores in groups that ingested bLf (0.6 or 2 g daily) were significantly lower than in the placebo group after 2 weeks of treatment [56].

We have examined cellular internalization of recombinant hLf into Caco-2 cells. Recombinant hLf entered the cell only from the apical side, and once internalized, hLf was localized inside the nucleus [41]. In fact, localization of hLf to the nucleus has been reported previously [57]. Although localization to the nucleus may implicate a direct involvement of hLf in signal transduction as a transcription factor through interacting with chromosomal genes, it is also possible that the SI-LfR transduces signals without transporting Lf inside the cell, which still remains to be elucidated. These studies indicate that hLf as well as bLf may modulate intestinal mucosal immunity through SI-LfR-mediated signaling, which could promote a systemic immune response against a vast variety of diseases.

Distribution in the body

Since the identification of human and mouse SI-LfR, the mouse has been used as a model to explore the tissue distribution of SI-LfR and intelectin, the homologue of SI-LfR. More recent work has advanced our knowledge of the distribution of SI-LfR messenger RNA (mRNA) and protein expression in various human and mouse tissues. Screening of adult human and fetal tissues for SI-LfR mRNA revealed that in adult tissues, salivary gland, heart, skeletal muscle, testes, adrenal gland and pancreas express an abundance of SI-LfR and other tissues to a lesser extent. In fetal tissue, SI-LfR was predominately and exclusively expressed in high amounts in the small intestine [5]. Similar screening of mouse tissues has demonstrated that SI-LfR is expressed in small intestine, testes, spleen and lungs. At the protein level, immunohistochemical results show SI-LfR expression in the digestive tract (esophagus, stomach, small intestine, large intestine, pancreas and renal cortex), nervous system (ganglia, cerebellum, hippocampus, hypothalamus, pituitary gland and spinal cord), reproductive system (ovaries, placenta) and other tissues (such as heart, lung, liver) in adult mice [32]. Localization of intelectin by in situ hybridization revealed mRNA expression in Paneth cells, which are situated in the lower region of the adult mouse intestine [33]. The expression pattern of SI-LfRs in the intestine supports the notion that SI-LfRs may play a role in iron uptake and antimicrobial activities due to its expression in the small intestine and Paneth cells, respectively. Recently, to explore whether SI-LfRs play a role in infant iron uptake, we examined the localization of SI-LfRs in the developing mouse intestine during early fetal and neonatal life. The development of mouse fetal intestine begins at gestation day (GD) 7.5; however, it is not until GD 18.5 that the fetal gut begins to resemble mature tissue, although it is still poorly differentiated. Our work demonstrates that SI-LfR protein is expressed on the luminal side of the nascent villi at GD 18.5, before birth. Further examina-

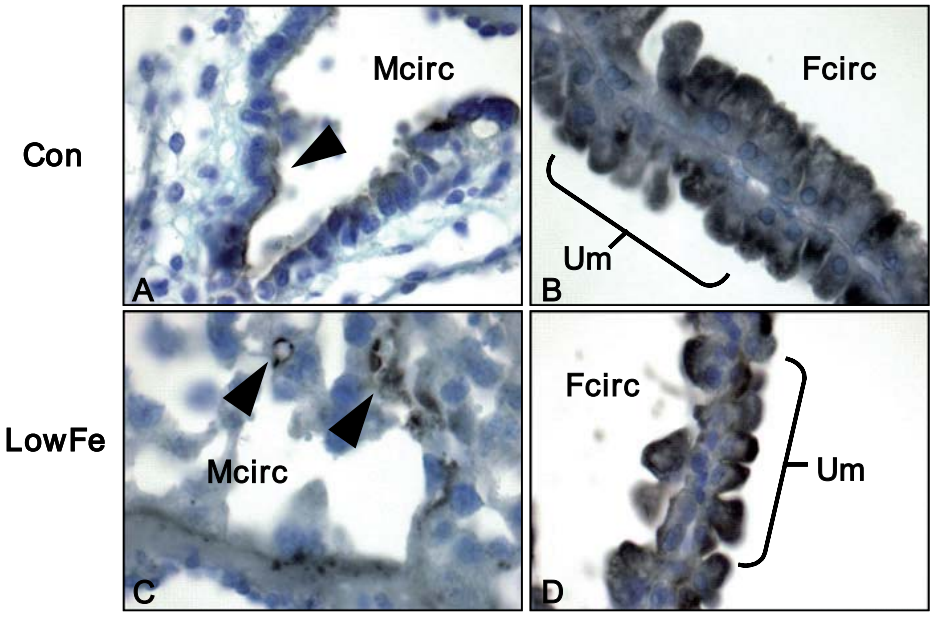

Figure 2. Serial sections of mature gestational day (GD) 18.5 mouse placenta assessed for SI-LfR localization by immunohistochemistry. Mice were fed an egg white-based diet containing control Fe, 75 mg Fe/kg, or LowFe diet containing 8 mg Fe/kg prior to breeding and throughout pregnancy. A and C, GD 18.5 placenta tissue stained positive for SI-LfR, associated with the plasma membrane (arrowheads) at the main stem villous region associated with the maternal circulation (Mcirc); however, the pattern of staining in LowFe tissue appeared clustered (arrowhead) in a similar region. B and D, SI-LfR expression appeared coupled to the fetal circulation (Fcirc) via the umbilical cord (Um).

tion of SI-LfRs demonstrated that localization of SI-LfRs did change but not with during postnatal development (age) or maternal iron status. SI-LfR protein expression was also present in the crypt region at postnatal day (PD) 20. This localization in the lower villous region is similar to that observed for intelectin mRNA, which is associated with differentiated Paneth cells. Seeking to understand the mechanisms mediating SI-LfR expression in the intestine, one could speculate that transcriptional control by transcription factors, such as Math1, might be observed. Math1 is a basic helix-loop-helix transcription factor which is expressed in the early gut, and loss of its function results in a reduction in Paneth cells [58]. Therefore, Math-1 may be an upstream regulator of SI-LfR expression associated with its expression in Paneth cells. However, further studies on mouse intestine are needed to provide a better understanding of the molecular events regulating the synthesis and function of SI-LfRs located in different regions of the intestine.

Much less is known about the expression and possible roles of SI-LfRs in the mammalian placenta. The placenta is derived from extra-embryonic tissue and is critical for the survival of the mammalian embryo [59, 60]. Current research suggests that SI-LfR protein is expressed in pre-implantation embryos (specifically in the extra-embryonic tissue), and uptake mechanisms for Lf involved are receptor-mediated [61]. We have determined that SI-LfR protein expression is localized in regions that interface both maternal and fetal circulation of the placenta (fig. 2A–D). Unlike the observations in fetal intestinal tissue, we did note a difference in localization of SI-LfR in placental tissue with maternal iron status (fig. 2A and C). The significance of this observation has not yet been determined. Research is currently under way to examine the cellular events associated with the Lf-SI-LfR interaction in the placenta. Such data may help to facilitate our understanding of the multifunctionality of SI-LfR associated with various tissues

Other mammalian LfRs

Liver LfR1, LDL receptor-related protein (LRP)

When hLf is injected intravenously, it is rapidly removed from the circulation of mice [62], rats [63], rabbits [64] and humans [65]. This rapid clearance was found to be mainly due to an association of hLf to the liver. Rat parenchymal liver cells have been found to bind hLf, which inhibits LRP-mediated uptake of apolipoprotein E (apo-E)-bearing lipoproteins by the liver [66]. Bound hLf was internalized into the cells and transported to a lysosomal compartment, where it is degraded [67]. Removal of 14 N-terminal amino acids of hLf by aminopeptidase M (APM-hLf), an exopeptidase that cleaves amino acids from the N-terminal end of a protein until it gets to a proline residue, enhanced its affinity for parenchymal liver cells [66]. On the other hand, selective modification of arginine residues with 1,2-cyclohexanedione (CHD) markedly reduced the affinity of hLf to parenchymal liver cells [66]. APM-hLf, which does not have the four-arginine cluster at positions 2–5, significantly inhibited LRP-mediated uptake of VLDL (very low density lipoprotein), but CHD-APM-hLf could not, suggesting that arginine residues in hLf are involved in the interaction with LRP but not the four-arginine cluster at positions

2–5 [66]. Furthermore, it has been reported that bLf can interact with the extracellular domain of LRP [68]. It has also been reported that bLf binds to both LRP1 and LRP2. These studies are informative from a biochemical perspective; however, their physiological relevance is questionable, as a rat model was used to assess the binding characteristics of hLf to liver. Rats do not produce Lf, and therefore seeking insight into the physiological functions of Lf by using a rat model may not be appropriate. Keeping this caution in mind, however, there are some recent interesting reports which seem relevant to review here.

LRP is a membrane glycoprotein that is abundantly expressed on hepatocytes, neurons, smooth muscle cells and fibroblasts [69]. It consists of an extracellular 515-kDa heavy chain and an 85-kDa light chain that spans the cell membrane. The extracellular domain of LRP contains four ligand-binding clusters denoted I–IV. It is now known that hLf interacts with the second and fourth cluster of class A cystein-rich repeats of human LRP [70]. LRP is known as an endocytotic receptor and participates in hepatocyte uptake of lipoproteins containing triglycerides and cholesterol. However, the broad range of its ligand diversity and the lethality of LRP knockout mice indicate that LRP is involved in diverse physiological and pathological processes other than just lipoprotein metabolism. These processes may include cell migration, fibrinolysis, thrombosis and atheroscleosis [69].

It has been reported that removal of bLf from plasma is mediated by LRP [71]. Translocation of bLf across the blood-brain barrier was also reported to be mediated by LRP [72]. LRP was clearly required, as a functional LfR, for bLf-induced fibroblastic contractility, since suppressing LRP expression with an antisense oligonucleotide blocked the collagen gel contractile promoting ability of bLf to human fibroblasts [73]. Collagen gel contraction is a phenomenon that, in three-dimensionally cultured fibroblasts, reorganizes the surrounding collagen gel matrix into a more dense and compact structure. This phenomenon is considered to mimic the reorganization of the collagen matrix that accompanies wound healing and pathological tissue contracture [74]. The effect of bLf in inducing fibroblastic contraction was found to be associated with increased phosphorylation of extra-cellular signal-related kinase (ERK) and myosin light-chain (MLC) kinase, which was decreased by suppressing LRP expression. However, suppressing LRP expression did not affect the specific binding of bLf to human fibroblasts [73]. Thus, for the initial binding events, an alternative Lf-binding molecule may be present on the surface of human fibroblasts, and LRP might act as a co-receptor in mediating the signaling pathway.

It has recently been shown that bLf has anabolic effects on bone; bLf stimulates mitogenesis, differentiation and survival of osteoblasts [75]. These effects have subsequently been reported to be mediated by signaling through LRP1 to p42/44 mitogen-activated protein kinases (MAPKs) [76]. These authors first confirmed that bLf bound to both primary rat osteoblastic cells and human osteoblastic SaOS2 cells by a conventional competitive binding assay and that binding was inhibited by excess unlabeled bLf. Primary rat osteoblastic cells were able to internalize bLf in an LRP-dependent manner. However, blocking of endocytosis did not abrogate the mitogenic effect of bLf, suggesting that internalization of bLf by osteoblastic cells is not required for activation of mitogenic signaling and that the endocytic function of LRP is independent of its signaling function. The mitogenic response of LRP-null fibroblasts to bLf was substantially reduced, but a weak mitogenic effect of bLf still remained. Therefore, all mitogenic activities of bLf are not sufficiently explained by mediation via LRP.

Liver LfR2 (LV-LfR2, asialoglycoprotein receptor)

Other researchers have investigated the rat hepatocyte LfR using bLf as a ligand. Holo- and apo-bLf were found to bind to hepatocytes in a nearly identical manner [77]. Optimum pH was at 7–8. Both apo- and holo-bLf competed with ^{125}I-holo-bLf, and thus apo- and holo-bLf bind to the same sites on the surface of hepatocytes. Furthermore, Ca^{2+}-independent low-affinity LV-LfR1 and Ca^{2+}-dependent high-affinity LV-LfR2 were observed. The specificity of Ca^{2+}-independent LV-LfR1 binding was tested by competition with strongly cationic proteins such as protamine sulfate (pI 12.1), lactoperoxidase (pI 9.6), lysozyme (pI 11.4) or cytochrome c (pI 10.6). Protamine sulfate and lactoperoxidase profoundly reduced holo-bLf binding, but lysozyme and cytochrome c did not. Therefore, binding of bLf to Ca^{2+}-independent LV-LfR1 is not due solely to an electrostatic interaction between bLf and acidic groups on Ca^{2+}-independent LV-LfR1. This Ca^{2+}-independent low-affinity LV-LfR1 is likely to be LRP. Subsequently, hepatocytes have been shown to endocytose bLf, but not to distinguish between apo and holo forms [78]. Endocytosis of bLf is only mediated by the Ca^{2+}-dependent LV-LfR2. Rat hepatocytes are known to internalize macromolecules by clathrin-dependent and -independent pathways [79–81]. Blocking of the clathrin-dependent pathway by using hyperosmotic medium almost completely shut down endocytosis of bLf by hepatocytes, indicating that endocytosis of bLf is primarily clathrin-dependent. Furthermore, derivatization of lysine residues on bLf with fluorescein isothiocyanate (FITC) or arginine residues with cyclohexane-1,2-dione (CHD) did not affect bLf binding and uptake by hepatocytes. This suggests that the cationic nature of bLf is not

needed for the interaction of bLf with Ca^{2+}-dependent LV-LfR2. The rat Ca^{2+}-dependent LV-LfR2 was later identified to be the RHL-1 subunit of the asialoglycoprotein (ASGP) receptor [82]. The RHL-1 subunit has lectin activity, but deglycosylated bLf was able to compete with unmodified bLf for binding to RHL-1 [83]. Studies on the direct interaction between bLf and ASGP receptors indicated that bLf binding to the ASGP receptor requires the carbohydrate-recognition domain of the receptor to be in the active configuration [84]. The primary event after the interaction of bLf with the ASGP receptor is not yet known.

Lymphocyte LfR (LC-LfR)

Considerable attention has been paid to the LC-LfR because of its potential role in maturation and function of cells of the immune system. When lymphocytes were stimulated with phytohemagglutinin, hLf was able to bind to the cells while resting cells did not show detectable binding [85]. Mitogen-activated lymphocytes were found to express the LC-LfR, which was visualized by hLf ligand blotting. The size of the LC-LfR was ~105 kDa. The degree of iron saturation did not affect binding kinetics, but growth stimulation as examined by thymidine incorporation into phytohemagglutinin-stimulated lymphocytes was dependent upon iron saturation of hLf; the lower the iron saturation, the higher the effect on thymidine incorporation. Purification of the LC-LfR was successful by using an immobilized anti-Lf-immunoglobulin (Ig) G Sepharose column. Binding activity of purified LC-LfR was retained and found to be pH-dependent with optimal binding between pH 6.5 and 7.5. The hLf recognition site of the LC-LfR has been investigated in detail using tryptic fragments of hLf [86] and derivatives that were blocked at specific lysine residues of hLf [87]. The N-tryptic fragment (residues 4–281) was recognized by the LC-LfR with the same affinity as that of native hLf and inhibited binding of ^{125}I-hLf to the LC-LfR by 50–60%. The number of binding sites for the N-tryptic fragment was half of that of native hLf. On the other hand, neither N-terminal domain II (residues 91–257) nor a 50-kDa C-terminal tryptic fragment (residues 282–703) bound to the LC-LfR and did not inhibit binding of ^{125}I-hLf to the LC-LfR. These results indicate that the binding site on hLf for the LC-LfR is located in N-terminal domain I, which appears to be recognized by only half of the binding sites for native hLf [86]. It is thus possible that there are two kinds of LC-LfR or two binding sites in one LC-LfR, and that one could interact with just the N-tryptic fragment but the other requires the entire configuration of hLf. Subsequently, the hLf derivatives SASD-hLf and SANPAH-hLf were synthesized to further determine the binding site on hLf. SASD-hLf, which is blocked on lysine-74 of hLf, did not bind to the LC-LfR, whereas SANPAH-hLf, which is blocked on lysine-281 of hLf, bound to it. Molecular modeling showed that SASD could mask residues 4–6 and two loop-containing regions of hLf (residues 28–34 and 38–45), suggesting that these regions are involved in interactions between hLf and the LC-LfR [87].

A monoclonal antibody has subsequently been raised against the soluble LC-LfR purified from cell culture supernatant of the human lymphoblastic Jurkat T cell line [88]. The monoclonal antibody recognized both the soluble and the membrane LC-LfR and inhibited binding of hLf to Jurkat cells and to activated human peripheral lymphocytes. The LC-LfR was localized both inside and at the cell surface of Jurkat cells. Jurkat cells internalized hLf, which then localized to endosome vesicles, and was released [89]. During each round of endocytosis, 30–40% of internalized hLf was degraded. Structure-affinity studies using several Lfs showed that hydrogen bonding is a significant binding factor for the interaction between Lf and LC-LfR [90].

B lymphocytes have been found to bind [91], incorporate and release hLf [92]. It has been suggested that both the N-terminal basic region and the basic characteristics of the whole molecule of hLf are likely to contribute to the interaction with B lymphocytes [93]. Thus, maturation of immature B and T lymphocytes, which is mediated by the LfR, is suggested to be a major biological property of hLf. In fact, hLf has been shown to accelerate the differentiation process of immature B- and T-lymphocytes [94]. When incubated with hLf, the expression of CD4 antigen on the cell surface of $CD4^-$ $CD8^-$ murine thymocytes was strongly enhanced, suggesting acquisition of the characteristic for the helper cell phenotype. Similarly, hLf has been shown to stimulate the maturation process of murine splenic B cells [95]. It has been observed in Jurkat cells that the effect of hLf on CD4 surface density was dose-dependent and that CD4 expression was upregulated with an optimal response after 48 h of incubation with hLf [96]. The early stages of signal transduction leading to the CD4 expression in Jurkat cells were also investigated. Tyrosine phosphorylation of numerous proteins was induced by hLf. The activity of MAPK, a key regulatory enzyme involved in the differentiation and proliferation process, also increased rapidly and transiently towards the myelin basic protein, one of its specific substrates. Tyrosine kinases were found to be located upstream from MAPK activation and CD4 expression since tyrosine kinase inhibitor blocked CD4 expression and MAPK activation by hLf. It has been confirmed that MAPK is required for the signal transduction induced by hLf, leading to CD4 upregulation since a selective inhibitor of the MAPK-activating enzyme blocked the effect of hLf. The lymphocyte-specific protein kinase p56lck was also involved in the pathway leading to maturation of Jurkat cells by hLf. Although some molecular mechanisms have

been revealed, our understanding of the direct interactions of hLf and the LC-LfR through different pathways is still limited.

Monocyte (macrophage) LfR (MC-LfR)

Mouse peritoneal macrophages was the first cell type reported to express LfR in mammals [97]. These peritoneal macrophages were found to take up hLf in vitro, whereas Tf was not ingested [98]. In addition, the interaction between hLf and MC-LfR was suggested to be involved in iron turnover since Fe was transferred from hLf to ferritin. In a murine macrophage-like cell line, P388D1, hLf did not donate iron to ferritin, but enhanced thymidine incorporation, suggesting that iron donation to the cell is not the only role of the interaction between hLf and MC-LfR [99]. Human monocytes were also shown to bind hLf in a reversible, saturable and specific manner [100]. Specificity was demonstrated by competitive binding studies with hTf. The properties of hLf were studied after its interaction with human monocytes [101]. Rebinding of this hLf to the MC-LfR was impaired, and its isoelectric point was slightly lower. In contrast, the molecular weight, antigenic and iron-binding properties of hLf were preserved. These findings indicate that hLf molecules cannot operate in a cyclic manner to deposit iron. The MC-LfR therefore is most likely involved in the effects of hLf on the inflammatory and immune responses of animals [102]. In fact, hLf has been shown to regulate the production of pro-inflammatory cytokines in mononuclear cells [103, 104]. This seems to be due to the activation of the cytotoxicity of NK cells [105]. Furthermore, a protective function of Lf against sublethal doses of lipopolysaccharide (LPS) has been reported in mice [106, 107]. In addition, a protective effect of Lf feeding against endotoxin lethal shock in germfree piglets has been described [108].

Monocytes are known to express CD14, an LPS receptor that activates the immune system. It has been shown that hLf interacts directly with soluble CD14 (sCD14) and protects animals from septic shock induced by LPS [109]. The kinetic binding parameters of hLf to sCD14 have been investigated, and it was found that hLf bound to sCD14 with high affinity (K_d = 16 ± 7 nM). It has also been shown that hLf binds to sCD14 complexed to LPS or lipid A-2-keto-3-deoxyoctanoic acid-heptose, but with different binding properties. CD14 is a 55-kDa glycoprotein that exists both as a soluble protein found in serum at concentrations of 2–6 μg/ml [110] and as a GPI-anchored protein (mCD14) on the surface of monocytes-macrophages [111]. It has also been found that hLf significantly inhibits expression of E-selectin (endothelial-leukocyte adhesion molecule 1) and of ICAM-1 (intercellular adhesion molecule 1) at the surface of human umbilical vein endothelial cells. These observations suggest that the anti-inflammatory effects of hLf are due partly to its ability to interact with sCD14 and with the sCD14 complexed to LPS, thus modifying the activation of endothelial cells. It is therefore likely that CD14 is one of the MC-LfRs.

THP-1, a human monocytic leukemia cell line, has been widely used in LfR research. The binding capacity for Lf was found to increase as the cells differentiated into macrophages, and was not affected by iron saturation of hLf [112]. Binding was primarily mediated by its protein component, but a short oligosaccharide structure of hLf is also recognized by the MC-LfRs. At least four species of hLf-binding proteins, 35, 35-37, 50 and 80 kDa, respectively, were found in the membrane of THP-1 cells [113], and the N-terminal amino acid sequence of the 50-kDa protein was recently determined [114]. It appears to be a novel sequence, and the gene has not yet been cloned. An antibody was raised against the chemically synthesized peptide composed of the N-terminal amino acid sequence and was found to bind to 50-, 60- and 80-kDa proteins, suggesting that these three proteins have an identical N-terminal sequence. The interaction of hLf with THP-1cells has been shown to downregulate LPS-induced cytokines. These findings are likely to be explained by the interference by NF-kappa B that is activated by internalized and nuclear localized hLf [115].

Platelet LfR (PL-LfR)

LfRs have also been found in non-activated human platelets and have been characterized to some extent. Optimum binding was attained at 37 °C, pH 7.0–7.4 with an apparent K_d of 6.0 pM for the higher-affinity binding site [116]. The KRDS tetrapeptide, which corresponds to loop 39–42 of the hLf molecule, was found to be an integral part of the PL-LfR binding site, and to inhibit platelet aggregation [116, 117]. The 50% inhibition (IC_{50}) of the N-terminal tryptic fragment was 2 μM, and the C-terminal tryptic hLf fragment did not show any inhibition. The IC_{50} of the KRDS was 500 μM, and that of hLf was 30 nM. On the other hand, a synthetic octodecapeptide CFQWQRNMRKVRGPPVSC (residues 20–37 of hLf), which corresponds to one of the two external loops (residues 28–34 and 39–42), inhibits ADP-induced platelet aggregation with an IC_{50} of 20 μM [118]. The two external loops were previously identified as the binding site for the PL-LfR [87]. However, the octodecapeptide inhibits platelet aggregation much more potently than KRDS, suggesting that the accessible hLf loop at 28–34 plays a key role in the inhibition of platelet aggregation. The inhibitory effect of hLf on platelet aggregation was observed at concentrations down to 5 nM [118], which is not far from the physiological concentration of hLf in blood (1–3 nM) and within the range of pathological cases when hLf is locally released from leukocyte sec-

ondary granules [119, 120]. The PL-LfR was purified, and its immunological and physicochemical properties were found to be very similar to those of the LC-LfR [118]. The LfR was also present in megakaryocytes, but only in a subpopulation of the largest cells, and a mouse monoclonal antibody, produced against the LC-LfR, allowed its characterization as a 105-kDa protein on Western blots [121]. Thus, the PL-LfR is likely to be identical to the LC-LfR.

Mammary epithelial cell LfRs (ME-LfR)

The presence of ME-LfR on epithelial SV-40 immortalized cell lines derived from non-malignant human breast, benign mastopathies, oncogene-transformed cells and breast carcinomas was reported by Rochard et al. [122]. The ME-LfR was present at the surface of the different cell lines as demonstrated by flow cytometry using two different fluorescein-labeling methods. The binding parameters were of the same order of magnitude as those determined for activated lymphocytes. Since ME-LfR expression was not different among malignant and non-malignant or oncogene-transformed cells, the ME-LfR cannot be considered as a marker of tumor progression. The MAC-T bovine mammary epithelial cell line has been found to interact with bLf, which was not inhibited by excess bTf [123]. This interaction between bLf and MAC-T cells has been suggested to lead to inhibition of cell proliferation [124]. Inhibition of proliferation of tumor cells by hLf has been suggested to reduce tumor growth and inhibit experimental metastasis [125]. In fact, hLf inhibits in vitro growth arrest of breast epithelial cancer cells at the G1 to S transition of the cell cycle by modulating the expression and activity of key regulatory proteins [126].

Respiratory epithelial cell LfR (RE-LfR)

Immortalized respiratory epithelial cells from normal human bronchial epithelium, BEAS-2B, have been reported to express a LfR (RE-LfR), which was upregulated when exposed to iron or vanadium [127]. Although calcium or magnesium did not enhance RE-LfR levels, chelating those divalent ions by EGTA decreased binding of Lf (species are not specified in this paper) to the RE-LfR, suggesting that binding is calcium-dependent. Cycloheximide, a protein synthesis inhibitor, did not block RE-LfR upregulation by metals, and neither did actinomycin D, an inhibitor of RNA polymerase movement. These results suggest that posttranslational modification of the protein or translocation could contribute to upregulation of RE-LfR after metal exposure. These characteristics are similar to the LV-LfR2 [128]. Because of the upregulation of RE-LfR expression by metals, it has been postulated that RE-LfR may be involved in decreasing oxidative stress in the lower respiratory tract by complexing catalytically active metals.

Brain LfR (BR-LfR)

In Parkinson's disease, neurons have been found to accumulate high concentrations of hLf [129]. Although mLf transcripts were found to be upregulated in a model for Parkinson's disease [130], the large increase in Lf observed in Parkinson's disease cannot be explained [129]. Thus, it was hypothesized that Lf crosses the blood-brain barrier through a brain LfR (BR-LfR)-mediated pathway. Differentiated bovine brain capillary endothelial cells have been shown to bind bLf at high (K_d = 40 nM; n = 90,000 sites/cell) and low (K_d = 2 μM; n = 900,000 sites/cell) affinity binding sites [72]. Non-differentiated cells exhibited only low-affinity binding sites, and only differentiated cells were able to internalize bLf, suggesting that BR-LfR is expressed during cell differentiation. The BR-LfR-mediated transport of bLf was found to be unidirectional and occurred without apparent intra-endothelial degradation. An antagonist to LRP was able to inhibit 70% of bLf transport, suggesting that LRP may be the BR-LfR.

Summary and future trends

Characteristics of LfRs appear to vary considerably among species, tissues and cell types. The SI-LfR is also called intelectin. With the first small intestinal LfR cloned, many questions arise, e. g. whether it is the same intelectin-like cDNA in all other species, such as piglet, rabbit, macaque and bovine; whether the GPI anchor is the only anchor or whether other transmembrane proteins are associated with the SI-LfR in locating to the surface of epithelium, to endocytose Lf or to transduce the signals inside the cell; whether the SI-LfR expressed in other tissues has the same primary function or not; what regulates the expression of the SI-LfR, iron status or infectious status. It is conceivable that many exciting new features such as those listed above will be unveiled in the near future. The rat liver LfRs are now known to be the same as the lower-affinity LRP and higher-affinity ASGP receptors. The interaction between Lf and LRP induces fibroblast contractility and bone anabolism. It is not yet known whether LRP and ASGP receptors are expressed in human infant small intestine, which should be addressed in the future. One of the MC-LfRs is CD14, which is likely to regulate pro-inflammatory responses. There appears to be another type of MC-LfR which still needs to be elucidated at the molecular level. The LC-LfRs in the circulation system are involved in signal transduction, leading to maturation of lymphocytes that is likely to enhance immune function systemically. Molecular information regarding LC-LfRs

is still not available. When molecular information for all these LfRs has been attained, it should be possible to elucidate the function and expression of different LfRs more accurately and to explore the true physiological functions of Lf and the LfRs.

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