Chinese Journal of Cancer Research 7(2): 79 - 85, 1995.

Basic Investigations

ROLE OF SIALIC ACID RESIDUES IN IRON BINDING BY HUMAN LACTOFERRIN - α*

Li Zhenpu 李振甫 Philip Furmanski[1]

Beijing Institute for Cancer Research, The School of Oncology, Beijing Medical University, Beijing 100034; [1]Department of Biology, New York University, USA

Lactoferrin (Lf - α) is a major constituent of the secondary granules of neutrophils, and is thought to be involved in bacteriostasis through high affinity binding of plasma Fe required for microbial growth. Lf - α is also the major Fe binding protein in milk. Fe bound to Lf - α is available for metabolic use, but the mechanism of Fe release from Lf - α is unknown.

Treatment of human Lf - α with neuraminidase to remove sialic acid residues reduced its ability to bind Fe and caused release of Fe already bound to the protein. Reduction in Fe binding capacity was dependent on time of exposure to and concentration of neuraminidase, and was not due to Lf - α loss or degradation. Readdition of sialic acid to desialylated Lf - α using α - 2, 6 - sialyltransferase restored Fe binding. The results suggest that sialylation - desialylation reactions could play a role in physiologic binding and release of Fe from the very high affinity binding sites of Lf - α.

Key words: Lactoferrin - α, Sialic acid, Fe binding.

Accepted December 28, 1994

* This work was supported by National Institutes of Health Grants (Ca - 33188, Ca - 43174 and Ca - 48077).

Lactoferrin (Lf) is a multifunctional glycoprotein expressed in high concentrations in human milk and in secondary or specific granules of neutrophils.[1] The major role of Lf is thought to be Fe binding, for nutritional or transport purposes in milk, or bacteriostasis, following release from neutrophils in response to inflammatory stimuli and chelation of Fe essential for bacterial growth.

Binding of Fe to Lf is 300X stronger than to transferrin (Tf). Release of Fe from Lf requires very low pH and high ionic strength[2] and Fe binding capacity is retained even by proteolytic fragments of Lf.[3] Despite this highly avid binding, Fe bound to Lf is available for metabolic use. However, the physiologic mechanisms involved in regulating Fe binding and release by Lf remain unknown.

Numerous other functions have been attributed to Lf, including several regulatory and enzymatic activities.[4] We have recently reported the existence of 3 functionally distinct isoforms of Lf: α, the traditional Fe - binding form; β and γ, two forms that do not bind Fe but possess potent RNase activity.[5] The structural basis for the various activities of Lf and the differences among the isoforms are not known. The 3 isoforms are very closely related, if not identical, physically,

chemically and antigenically. It has also been reported that the human Lf gene is present in the genome as a single copy.[6] Thus, the primary structures of these isoforms may be the same and differences in their function, including Fe binding, could be due to posttranslational modifications.

We have found that Lf - α differs from the non - Fe - binding forms, Lf - β and Lf - γ, in sialic acid content (manuscript in preparation). This prompted us to examine the role of sialic acid residues in Fe binding by Lf. The results reported here show that removal of sialic acid residues from Lf - α greatly reduces Fe binding activity and causes release of bound Fe. Readdition of the sialic acid residues restores Fe binding capacity. These findings offer additional insight into the mechanism of Fe binding by Lf - α, the structural basis for the different functional forms of the glycoprotein, and a possible physiologic mechanism for regulating Fe binding and Lf reutilization.

MATERIALS AND METHODS

Materials

Human milk Lf - α (L - 3639), neuraminidase (sialidase, E.C.3,2,1,18) purified from Clostridium perfringens (Type X, Soluble, N - 2133, 190 units/mg, < 0.002 units/mg protease activity; insolubilized, N - 4883, 25 units/mg), neuraminidase purified from Vibrio cholerae (Type Ⅱ, N - 6514, 12 units/mg), N - acetylneuraminic acid, cytidine 5 - monophospho - N - acetyl neuraminic acid - sodium salt (CMP - NANA), were obtained from Sigma Chemical Co. (St. Louis, MO). α - 2, 6 - Sialyltransferase (CMP - N - acetylneuraminate: β - D - galactosyl - 1, 4 - N - acetyl - β - D - glucosamine α - 2, 6 - N - acetylneuraminyltransferase, E.C.2.4.99.1) was obtained from Boehringer Mannheim Corp. (Indianapolis, IN) and Genzyme Corp. (Boston, MA). $^{59}FeCl_3$ (10 mCi/mg Fe) was obtained from Amersham Corp. (Arlington Heights, IL). All other reagents were of the highest purity commercially available.

Neuraminidase Treatment

Lf - α was dissolved at 1 mg/ml in 0.02 M phosphate buffer, pH 6.0, containing 1mM Ca acetate. To 200 μl of solution was added 0.2 U neuraminidase (or other enzyme concentrations, as indicated). The samples were incubated with continuous shaking in a 37℃ waterbath for 1 hr. In some instances, the tubes were then heated at 90℃ for 1 min to stop neuraminidase action.

Fe Binding

Samples of native or treated Lf - α, containing 1 μg of Lf - α, were added to 50 μl of 0.1 M $NaHCO_3$ in 0.15 M NaCl, 0.01 M phosphate, pH 7.2 (PBS). $^{59}FeCl_3$ (150,000 cpm, - 0.7μg Fe) was then added and the mixture incubated for 1 hr at 37℃. The sample was diluted to 0.5 ml with veronal buffer (5 mM Na barbital - HCl, 50 mM NaCl, pH 7.4). Bound and unbound ^{59}Fe were separated by chromatography on 1 × 2 cm columns of AGl - X8 resin (Bio - Rad Laboratories, Richmond, CA) eluted with veronal buffer. Fractions of 0.5 ml were collected and their radioactivity determined in a gamma counter to calculate protein - bound (eluted) Fe.

Fe Release

Lf - α (1 mg in 0.5 ml veronal buffer) was added to 0.5 ml of 0.1 M $NaHCO_3$ in PBS, followed by 10^7 cpm $^{59}FeCl_3$. The mixture was incubated for 1 hr at 37℃ with continuous shaking. The sample was then dialyzed against 0.02 M phosphate buffer, pH 6.0, containing 1 mM Ca acetate. Aliquots of 200 μl of the ^{59}Fe - Lf - α were incubated with 0.2 U of insolubilized neuraminidase or 0.2 U of insolubilized neuraminidase that had been inactivated by heating at 100℃ for 2 min. The samples were incubated at 37℃ with continuous shaking. At various times, 10 μl aliquots were removed, diluted with 0.5 ml ice - cold

veronal buffer, and centrifuged to remove the enzyme. Remaining bound ^{59} Fe was determined as described above.

Sialylation of Desialylated LF – α

Lf – α was desialylated with insolubilized neuraminidase (0.2 U enzyme/200 μg Lf – α) for 1 hr at 37℃ as described above and separated from the enzyme by centrifugation. Bovine serum albumin (0.2 mg) was added and the mixtures dialyzed overnight against 50 mM Na cacodylate buffer, pH 6.0. Then, 0.2 mU α – 2, 6 – sialyltransferase and 6 mμmoles CMP – NANA were added. The mixture was incubated at 37℃ for 24 hrs and then dialyzed against veronal buffer. Aliquots of the treated Lf – α were tested for Fe binding or sialic acid content.

Sialic Acid Determinations

The sialic acid content of the various preparations indicated was assayed by the periodate – resorcinol method. Briefly, 0.5 mg samples of protein in 0.5 ml veronal buffer were added to 0.1 ml of 0.04 M periodic acid. The solutions were incubated at 4℃ for 20 min, and 1.25 ml of resorcinol reagent added. The tubes were placed in an ice bath for 5 min, then in a boiling water bath for 15 min, and then cooled in tap water, tert – Butyl alcohol (1.25 ml) was added, the tubes vigorously mixed and incubated at 37℃ for 3 min. After cooling to room temperature, the OD at 630 nm was determined. Sialic acid contents were calculated by reference to standard curves made using egg N – acetylneuraminic acid.

RESULTS

The role of sialic acid in binding of Fe to LF – α was first examined by determining the effects of sialic acid removal using neuraminidase. As shown in Table 1, neuraminidase treatment of human mild Lf – α reduced Fe binding by > 90%. Heated neuraminidase had little to no effect. In numerous replicate experiments with several different preparations of Lf – α, neuraminidase treatment under these conditions reduced Fe binding by 65—92%.

Table 1. Effect of neuraminidase treatment on iron binding by human milk Lf – α

	cmp^{59} bound	% Fe binding
Lf, no neuraminidase	15,544	100
Lf + neuraminidase	1,235	8
Lf + heated neuraminidase	13,985	90
Neuraminidase alone	0	0

Buffer control (no Lf, no neuraminidase) value (961 cpm) subtracted from other determinations. Heated neuraminidase was incubated at 100℃ for 2 min.

Sialic acid determinations on native Lf – α gave a value of 6.25 nmoles/500 μg protein, or an average of 2.08 mol sialic acid/mol Lf – α, assuming a molecular weight of 80,000 for Lf – α, a value in general agreement with previous observations by others.[7,8] Following treatment with neuraminidase (0.5U/500μg Lf – α, 1 hr 37℃), sialic acid content was reduced to 0.05 mol/mol protein.

Neuraminidase – treated Lf – α mingrated as a

single band on SDS - PAGE with a mobility that was not noticeably different from that of native Lf - α. The intensity of the stained band on SDS - PAGE corresponding to intact Lf - α did not change appreciably following neuraminidase treatment. Treated and untreated Lf - α likewise gave single bands of the same intensity on isoelectric focussing gels with pI's of approximately 8.65 and 8.72, respectively. Fe binding by neuraminidase treated Lf - α was not increased by inclusion of ascorbic acid (4 mM) or nitriloacetic acid (100 mM) in the Fe - binding incubation mixture. The amounts of neuraminidase - treated and untreated Lf - α recovered from the ion exchange column used to separate free and bound ^{59}Fe were the same, as determined by OD_{280} (data not shown).

Thus, neuraminidase treatment of Lf - α removed almost all of the sialic acid of the protein and concomitantly reduced the Fe binding capacity, but without significant protein degradation or loss.

The parameters of neuraminidase effects on Lf - α binding of Fe were examined. Reduction of Fe binding capacity was related to neuraminidase concentration (Figure 1). At concentrations of 0.2 U neuraminidase added to 200 μg Lf - α, the loss of Fe binding activity was time dependent and essentially complete following 1 hr incubation (not shown). Neuraminidase purified from Vibrio cholerae gave the same results as that from Clostridium perfringens used in the experiments above, as did insolubilized Clostridium enzyme (see below).

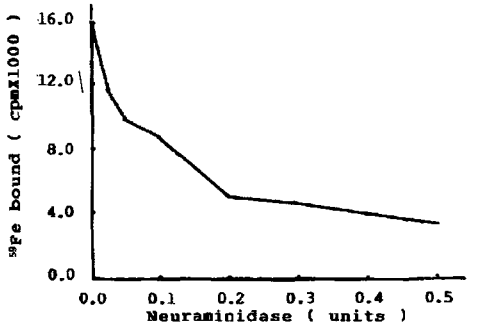

Fig. 1. Effects of varying concentrations of neuraminidase on the Fe - binding activity of human Lf - α.

To determine whether removal of sialic acids would cause release of Lf - α - bound Fe, Lf - α was preloaded with ^{59}Fe in the presence of $NaHCO_3$, treated with insolubilized neuraminidase and retention of ^{59}Fe determined by ion exchange chromatography. As shown in Figure 2, neuraminidase caused release of Fe bound to Lf - α in a time (and concentration, not shown) dependent manner. The reaction was essentially complete under these conditions in 1 hr, with release of about 70% of bound Fe.

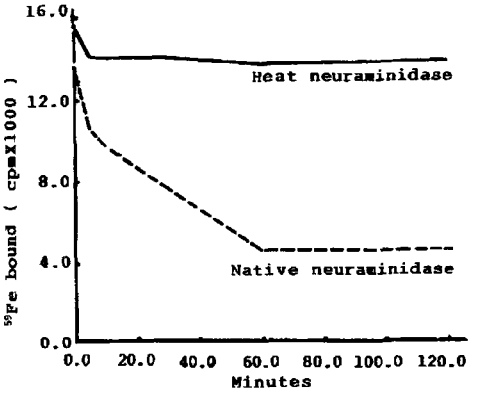

Fig. 2. Release of bound Fe from human Lf - α following treatment with native neuraminidase (0.2 units insolubilized enzyme) or heated neuraminidase (0.2 units insolubilized enzyme pretreated at 100℃ for 2 min).

Since removal of sialic acid residues reduced Fe binding capacity, it was of interest to determine whether readdition of sialic acid would restore the activity. Thus, neuraminidase - treated Lf - α was incubated with α - 2, 6 - sialyltransferase and CMP - NANA. Under these conditions, Lf - α was resialylated to about 50% of that in the initial, native preparation and an approximately equivalent proportion of the Fe binding capacity was regained (Table 2). Desialylated Lf - α treated with either α - 2, 6 - sialytransferase or CMP - NANA alone did not regain Fe binding activity (not shown). Treatment of native Lf - α with α - 2, 6 - sialytransferase and CMP - NANA did not affect Fe binding.

Table 2. Restoration of Fe – binding activity to desialylated Lf – α by α – 2, 6 – sialyltransferase and CMP – NANA

	cpm^{59} bound	% Fe binding	Sialic acid (mol/mol Lf)
Native Lf – α	13,659	100	2.08
Desialylated Lf – α	4,944	36	0.07
Desialylated Lf – α + α – 2, 6 – sialyltransferase and CMP – NANA	10,249	75	1.01

DISCUSSION

The studies reported here show that the sialic acid residues on Lf – α influence its ability to bind Fe. Treatment of Lf-α with neuraminidase caused a time and concentration dependent loss in the capacity to bind Fe, and caused release of Fe already bound to the molecule. That the enzymatic treatment caused these effects by removal of sialic acid residues, and not by protein degradation or other modifications, is evidenced by the facts that: 1) several different forms of neuraminidase had the same effect on Fe binding; 2) the effects of the different neuraminidases were quantitative related to their sialidase activity and not to their (low) proteolytic activity; 3) neuraminidase – treated Lf – α exhibited no significant degradation as determined by SDS – PAGE and isoelectric focussing; and, 4) resialylation of the desialylated Lf – α restored Fe binding capacity.

The mechanism by which Lf – α releases bound Fe is unknown. The association constant for Fe binding to Lf – α is – 10,[20] and extreme conditions of acidic pH and high ionic strength are generally necessary to effect complete disociation.[2] Yet Lf – α taken up by macrophages releases its Fe intracellularly to become bound to ferritin. Winterbourn and Molloy[9] recently reported that myeloperoxidase – catalyzed production of strong oxidants, as might occur in stimulated neutrophils, can cause a reduction in the ability of Lf – α and Tf to bind Fe and cause loss of Fe from Tf and Lf – α, although to a much lesser degree from Lf – α. The *in vivo* significance of these findings is as yet unclear. On the other hand, regulation of Fe binding by Lf – α through sialylation – desialylation reactions, which occur under physiologic conditions, offers a reversible, efficient and gentle mechanism for Fe and Lf – α reutilization.

Removal of Fe from Lf – α by neuraminidase treatment was incomplete, with 8—35% of the Fe remaining bound to the protein. This occurred despite the fact that enzyme treatment removed > 95% of Lf – α siallic acid. It is thus possible that an additional factor, such as pH, ionic strength, oxidizing conditions, the presence of an acceptor high – affinity Fe binding protein such as ferritin, is necessary to achieve complete dissociation. It has also been reported that asialo – Tf prepared using neuraminidase or acid treatment is not equivalent in behavior on lectin affinity achromatography to asialo – Tf prepared by exposure to liver endothelial cells.[10] Thus, if desialylation is a mechanism for Fe removal from Lf – α *in vivo*. its accomplishment by liver cells or macrophages which bear receptors for Lf – α might be more efficient and lead to greater Fe release. Whether 1 or both of the average of 2 sialic acid residues/Lf – α molecule are necessary for Fe binding is unknown.

Similarly, treatment of asialo – Lf – α with sialyltransferase did not restore all Fe binding. However, in this circumstance, the amount of Fe

binding obtained was approximately related to the degree of resialylation of the Lf – α, suggesting that the conditions for resialylation might not have been optimal and that if full resialylation were achieved full restoration of Fe binding would have occurred.

The amino acid, glycan and crystallographic structures of Lf – α are known.[7,8,11,12] The molecule consists of 703 amino acid residues organized into 2 lobes each comprised of 2 domains. Each lobe contains a single binding site for Fe^{3+} together with a HCO_3^- or CO_3^{2-} anion, and an N – asparagine – linked glycan in homologous positions. The glycans are exposed on the surface of the molecule attached to asparagines 137 and 490. They consist of biantennary structures of the N – acetyllactosamine type. Human Lf's are characterized by α – 1, 3 – fucosylation as well as α – 1, 6 – fucosylation. Sialic acids are α – 2, 6 – linked, and are located on every antenna with mannose α – 1, 3 linked to the core, as well as occasionally on the α – 1, 6 – linked mannose antenna. Roberts, et al. reported forms of sow milk Lf differing in pI's, which were attributed to different degrees of sialylation.

The relationship between Fe binding and the glycan structure is not clear. Each Fe is bound to Lf by coordination to 4 amino acids: the phenolate oxygens of two tyrosines (positions 93, 191 and 447, 540 on the N and C lobes, respectively), the $N\varepsilon_2$ imidazole nitrogen of a histidine (residues 252 and 609) and the carboxylate oxygen of aspartic acid (residues 61 and 407).[12] Interestingly, the coordination site on each lobe for the anion required for Fe binding (HCO_3^- or CO_3^{2-}) is adjacent to an α – helix which contains the glycan – N – asparagine linkage. Fe binding could thus be influenced by sialic acid effects on anion binding. It is also possible that sialylation has an allosteric or conformational effect that influences the Fe binding site. Conformational changes appear to accompany Fe binding to members of the Tf/Lf protein family.

Sialic acid residues could play a role in the cyclic reutilization of Lf – α. Sialylated Lf – α released from neutrophilic granules might bind Fe, be cleared from the circulation by attachment to receptors on acceptor cells and desialylated intracellularly to release Fe. The desialylated apo – LF – α could then be taken up by neutrophils, resialylated and retained in their secondary granules for subsequent release. Receptors for Lf – α have been identified on monocytes and macrophages, and are of relatively low affinity and very high abundance.[13] The Lf – α receptor may be one for a family of neutrophil granule glycoproteins released during inflammatory responses. The binding site may be membrane – associated DNA. After binding to the receptor, Lf – α Fe is thought to be released into the cell. Van Snick and Masson reported that Fe depletion eliminated Lf binding to its receptor, although others have found that apo – Lf – α bound to the receptor almost as well as the Fe – saturated form. Lf – α uptake *in vivo* by liver, which is the major site of plasma clearance, was reported to be mediated by fucose receptors on hepatocytes, although Moguilevsky, et al. concluded that liver uptake was due to binding sites for cationic proteins on reticuloendothelial cells and not the galactose or fucose receptors on liver parenchymal cells.[14] Finally, Lf – α binding to a receptor on mouse peritoneal cells was reported to be unaffected by desialylation of the protein. Thus, whether sialylation plays this proposed role in *in vivo* release of bound Fe and recycling of Lf – α remains unknown.

The relationship between sialic acid residues and function of the related Fe binding protein Tf is also of interest in this context. Tf is the primary mediator of Fe transport into cells for metabolic purposes.[1] Tf is thought to be bound to its receptor and taken up into lysosomes where the low pH facilitates Fe release. But the pH of lysosomes may be inadequate to cause Fe release from Tf. Moreover, in Hela cells Tf is internalized into a non – lysosomal compartment. Thus, the precise mechanism of Fe release from Tf, like Lf, remains to be determined.

Tf in serum is heterogene due to differences in sialylation. Human Tf's with between 2 and 6 sialic acids/mo nave been reported.[15] Rat serum TF, with a r. cular weight of 76,500, may contain 2 or 3 siali acid residues/

molecule. Desialylated Tf's accumulate in sera of alcoholics or patients with iron-storage diseases.[16,17] Rat liver endothelial cells take up Tf via a specific receptor, desialylate the Tf and release it extracellularly, while retaining a portion of the Fe within the cell.[18] Thus, sialic acid residues could play a role in the binding and release of Fe by Tf. However, K562 cells take up Tf, retain the Fe and appear to release fully sialylated, apo-Tf for recycling.

Thus, the mechanisms involved in release of Fe from high - affinity binding proteins such as Lf - α and Tf remain unclear and may be complex. However, sialylation-desialylation offers an attractive and physiologically tenable mechanism for its accomplishment.

REFERENCES

1. Aisen P, Listowsky I. Iron transport and storage proteins. Ann Rev Biochem 1980; 49:357.
2. Moguilevsky N, Retegui LA, Masson PL. Comparison of human lactoferrin from milk and neutrophilic leucocytes. Relative molecular mass, isoelectric point, iron - binding properties and uptake by the liver. Biochem J 1985; 229: 353.
3. Bluard - Deconinck JM, Williams J, Evans RW, et al. Masson PL: Iron - binding fragments from the N - terminal and C - terminal regions of human lactoferrin. Biochem J 1978; 171:321.
4. Broxmeyer HE. Biomolecule - cell interaction and the regulation of myelopoiesis. Int J Cell Cloning 1986; 4: 378.
5. Furmanski P, Li ZP, Fortuna MB, et al. Multiple molecular forms of human lactoferrin. Identification of a class of lactoferrins that possess ribonuclease activity and lack iron - binding capacity. J Exptl Med 1989; 170: 415.
6. Wei X, Nuss J, Rado TA. Characterization of the complete cDNA sequence of human neutrophil lactoferrin and isolation of genomic clones (abstr). Blood 1988; 72: 155a.
7. Matsumoto A, Yoshima H, Takasaki S, et al. Structural study of the sugar chains of human lactoferrin: Finding of four novel complex - type asparagine - linked sugar chains. J Biochem 1982; 91:143.
8. Spik G, Coddeville B, Montreuil J: Comparative study of the primary structures of sero - , lacto - and ovotrans - ferrin glycans from different species. Biochimie 1988; 70: 1459.
9. Winterbourn CC, Molloy AL. Susceptibilities of lacto - ferrin and transferrin to myeloperoxidase - dependent loss of iron - binding capacity. Biochem J 1988; 250:613.
10. Irie S, Minguell JJ, Tavassoli M. Comparison of de - sialylation of rat transferrin by cellular and non - cellular methods. Biochem J 1989; 259:427.
11. Metz - Boutique M - H, Jolles J, Mazurier J, et al. Human lactotransferrin: Amino acid sequence and structural comparison with other transferrins. Eur J Biochem 1984; 145:659.
12. Anderson BF, Baker HM, Dodson EJ, et al. Structure of human lactoferrin at 3.2 A resolution. Proc Natl Acad Sci USA 1987; 84:1769.
13. Yamada Y, Amagasaki T, Jacobsen DW, et al. Lactoferrin binding by leukemia cell lines. Blood 1987; 70:264.
14. Moguilevsky N, Retegui LA, Courtoy J, et al. Uptake of lactoferrin by the liver. Ⅲ. Critical role of the protein moiety. Lab Invest 1984; 50:335.
15. Petren S, Vesterberg O. The N - acetylneuraminic acid content of five forms of human transferrin. Biochim Biophys Acta 1989; 994:161.
16. Regoeczi E, Chindemi PA, Debanne MT. Transferrin glycans: A possible link between alcoholism and hepatic siderosis. Alcoholism 1984; 8:287.
17. Storey EL, Anderson GJ, Mack U, et al. Desialylated transferrin as a serological marker of chronic excessive alcohol ingestion. Lancet 1987; 1:1292.
18. Irie S, Kishimoto T, Tavassoli M. Desialation of transferrin by rat liver endothelium. J Clin Invest 1988; 82:508.