Relationship of milk iron and the changing concentration of mammary tissue transferrin receptors during the course of lactation

Madeleine Sigman and Bo Lönnerdal

Departments of Nutrition and Internal Medicine, University of California, Davis, Davis, CA 95616

The concentration of iron in all mammalian milks falls during lactation while the infant's iron requirement increases. Little is known, however, about the entry of iron into milk. Recently, transferrin receptors have been identified on lactating rat mammary plasma membranes, which may regulate iron entry into mammary tissue, potentiating its availability for subsequent transport into milk. This study was conducted to determine what relationship exists between the declining concentration of milk iron and the transferrin receptor concentration during various stages of lactation. Minimal transferrin receptors were detected in nulliparous rats. Total mammary transferrin receptor content increased during early and mid-lactation while milk iron concentration decreased. The continued appearance of high levels of transferrin receptors throughout lactation, without a concomitant increase in milk iron concentration, suggests a need for iron for functions other than cellular growth or secretion into milk to meet infant needs.

Keywords: mammary gland, transferrin, transferrin receptors, milk, iron

Introduction

The concentration of iron in all mammalian milks declines during the course of lactation at the same time as the infant's demand for iron is high due to the rapid growth.[1] In humans, milk iron concentration has been shown to be remarkably similar when comparing not only different populations but also when comparing milk from women having varying iron status. Moderate maternal iron deficiency as well as high iron status does not appear to affect milk iron. These observations suggest that milk iron concentration is regulated by some as yet unknown mechanism(s).

Recent work, using either immunological or radiolabeling techniques, suggests a transferrin receptor (Tf-R) system may be responsible for iron entry into the secreting mammary epithelial cell.[2,3] Shulman et al.,[4] using radiolabeling techniques, demonstrated an increase in Tf-R activity in the epithelial cell during glandular development which occurs during pregnancy and lactation. Using immunochemical techniques, we isolated and characterized a transferrin receptor from plasma membranes of lactating rat mammary cells.[2] This study was conducted to determine the relationship of milk iron concentration relative to the concentrations of mammary Tf-R during the stages of lactation (early lactation at day 2, mid-lactation at day 14 and during involution at day 20).

Methods and materials

Animals

Twenty-eight female Sprague-Dawley rats were purchased from a commercial supplier (Bantin & Kingman, Inc., Fremont, CA). Seven were nulliparous; twenty-one were lactating with litters of 10 pups. On day 2, 14, or 20 of lactation, seven dams each were used.

Address correspondence, proofs, and reprint requests to Dr. Bo Lönnerdal, Department of Nutrition, University of California, Davis, CA 95616.
Dr. Sigman's current address is Pennsylvania State University, Dept. of Food Science, 111 Borland Laboratory, State College, PA 16802.
Received March 9, 1990; accepted for publication June 25, 1990.

Milk collection. To avoid the damage to mammary tissue that occurs in traditional milk collection procedures,[5] an internal milking procedure was developed. Five to 6 hours following separation from pups, lactating dams were anesthetized with CO_2 for 20 seconds and injected intramuscularly with oxytocin (12 IU). After 10 minutes, dams were reanesthetized with CO_2 for 1 min and decapitated. Skin covering the mammary tissue was teased away, exposing both tissue and blood supply. After piercing the galactophore, the milk was quickly collected into a disposable glass pipet. Both inguinal and thoracic glands were milked.

Preparation of mammary tissue. After milk collection, the inguinal glands were removed, weighed, and finely minced. Following the procedures of Seligman and Allen,[6] the tissue was homogenized in an ice-cold buffer (1:2) of 10 mM KPO_4, containing 150 mM NaCl, 0.005 mM phenylmethylsulfonyl fluoride, and 0.02% NaN_3, using a Janke & Kunkel Ultra-Turrax Tissumizer at 70 rpm, 3 to 5 bursts of 10 seconds, separated by 30 seconds of cooling. The mixture was centrifuged (Sorval RC-5B) at 2000 × g for 15 minutes and the supernatant respun at 30,000 × g for 90 min. The pellet was resuspended through a 22 gauge needle in homogenization buffer. Triton-X 100 reduced (Sigma Chemical Company, St. Louis, MO) was added to a final concentration of 1%. The mixture was kept at 4°C for 4 hours, with constant stirring, until solubilized. The mixture was centrifuged at 30,000 × g for 1 hour and the supernatant respun for an additional 30 minutes. The solubilized preparations were stored at −70°C until analyzed. Protein content was determined by a modified Lowry procedure.[7]

Analytical methods

Milk iron concentration. The iron concentration of milk was analyzed using flame absorption spectrophotometry (Instrumentation Laboratories 551, Wilmington, MA) as described earlier.[8] Rat milk lactose was determined by the enzymatic method of Dahlquist.[9]

Transferrin receptor concentration. Solubilized mammary homogenates were applied directly onto 0.45 μm nitrocellulose membrane (Schleicher and Schuell, Keene, NH) as described by Hawkes[10] using the PR 600 Slot Blot apparatus (Hoeffer Scientific Industries, San Francisco, CA). The membrane was placed in a blocking solution of 5% non-fat dry milk solids in Tris buffered saline (NFDMS-TBS), pH 7.5, containing 0.2% NaN_3. After blocking overnight at room temperature, the membrane was gently shaken in a 1:75 dilution of mouse monoclonal rat Tf-R antibody (MRC-OX26) (USA Bioproducts for Science, Inc., Indianapolis, IN) in 1% NFDMS-TBS for 2 hours, followed by four 5 minute washes with TBS-Tween and one 5 minute wash with TBS. Incubation of the nitrocellulose with alkaline phosphatase-conjugated anti-mouse IgG antibody (Promega, Madison, WI) in TBS (1:7500 dilution) was followed by three 5 minute washes with TBS-Tween and one 5 minute wash with TBS. Detection of the receptor was performed using the Proto-Blot^R color detection system by Promega Biotech., Madison, WI (substrates: nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate). Transferrin receptor concentrations were determined using the GS-370 data system attached to a GS-300 Scanning Densitometer (Hoeffer Scientific Instruments, San Francisco, CA).

Statistical analysis

One-factor analysis of variance (ANOVA) was used to analyze the data. Analysis of covariance was used to determine interrelationships between the variables. The Fisher LSD and/or Scheffe HSD tests were used to compare differences between groups.

Results

Milk iron concentration was significantly higher ($p < .002$) at d 2 of lactation than at either d 14 or d 20 (Figure 1a), while lactose concentration was significantly higher ($p < .01$) during mid-lactation than in early lactation (Table 1). The mammary gland weights and their percentage of body weight were significantly lower in the early stage of lactation (Table 1).

All measurements of receptor concentrations [Tf-R] were made using arbitrary units/μg protein as determined by scanning densitometry and are thus described as relative arbitrary units. As Grigor et al.[3] have described, the absolute amount of transferrin receptor protein within the rat mammary tissue is very low, making it technically difficult to obtain enough receptor protein for use as a standard.

Minimal amounts of Tf-R were found in non-lactating tissue (Figure 2), while mammary homogenates from pregnant dams showed a significant amount of Tf-R. The relative [Tf-R]/μg protein was significantly higher ($p < .0001$) in early lactation than at either day 14 or 20 of lactation (Figure 1b). However, due to the larger gland sizes, total gland Tf-R content was higher in mid- and late-lactation (Figure 1c). The ratio of milk iron concentration to relative [Tf-R] was not significantly different between stages (Figure 1d).

Using one-factor ANOVA, a significant effect of stage of lactation on all parameters: milk iron concentration, relative [Tf-R], and total receptor content, was demonstrated (Table 2). When the effect of stage on milk iron was controlled, a significant relationship between relative [Tf-R] ($p < .001$), and Tf-R content, on milk iron was seen ($p < .01$).

Discussion

The procedure normally used to collect rat milk has been shown to affect milk composition.[5] The internal milking procedure we developed and used in this study did not appear to affect the values normally seen for lactose and iron concentrations in rat milk collected

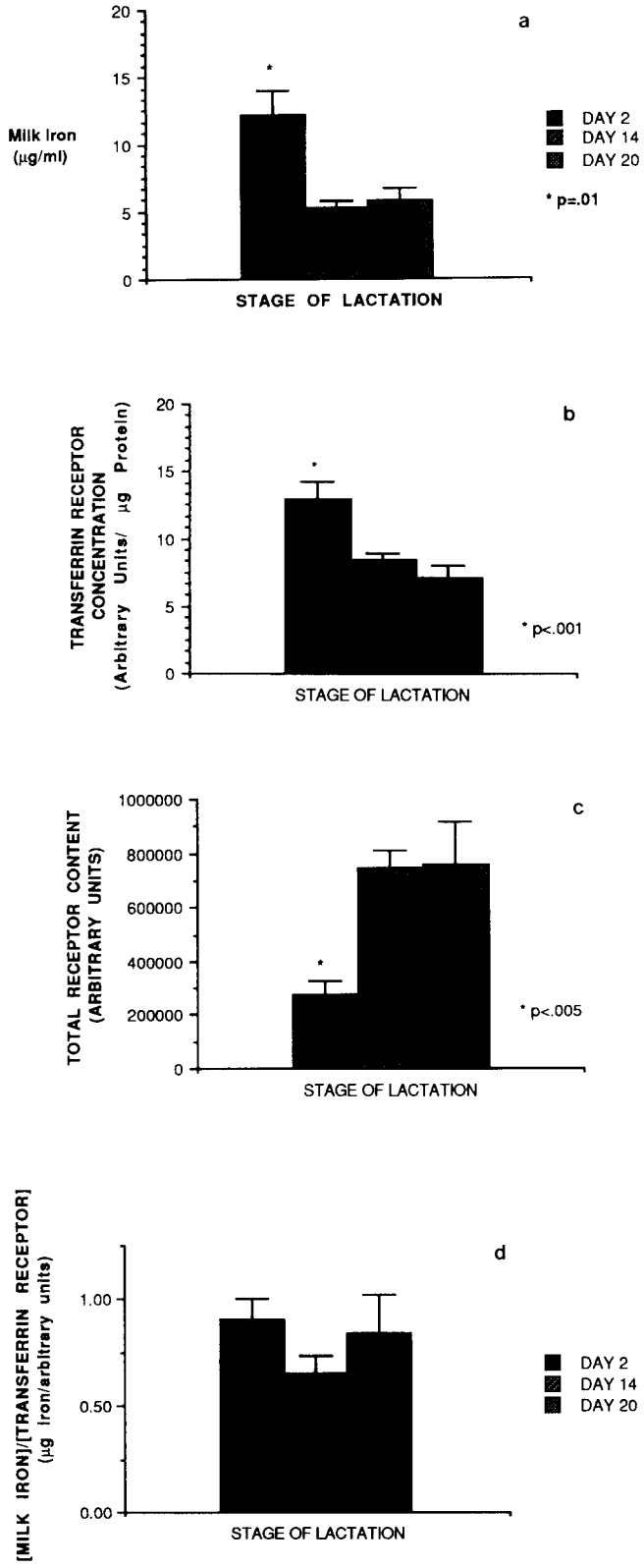

Figure 1 (a) Milk iron concentrations during lactation. Values represent means ± SEM (n = 5–7 animals/group). (b) Mammary gland transferrin receptor concentration during lactation. Values represent means ± SEM. (n = 5–7 animals/group). (c) Total mammary gland transferrin receptor content during lactation. Values represent means ± SEM. (n = 5–7 animals/group). (d) Ratio of [Milk iron] to relative [Transferrin receptor] during lactation. Values represent means ± SEM. (n = 5–7 animals/group).

using a single sample external collection procedure,[8] thus validating the use of our procedure.

There is a pronounced increase from pre-pregnancy to mid-lactation in relative [Tf-R] within the mammary tissue, as can be seen on the nitrocellulose membrane (Figure 2). These results agree with those obtained by others[3,4] using radiolabeling techniques for the determination of Tf-R activity on the secreting epithelial cells. However, while Grigor et al.[3] observed increased Tf-R activity from early to mid-lactation, we observed a decrease in the relative concentration of Tf-R for the same period. Only when we accounted for increased glandular protein content did we observe an increase in total Tf-R content.

Glandular development (mammogenesis) in the rat begins in pregnancy and continues until day 5 of lactation.[11] Although most of the growth is due to proliferation of epithelial cells, there are substantial increases in the stromal components of the gland as well.[12] Requirements for iron by either of these glandular constituents can be high during this phase.

Iron, which is essential for normal growth and metabolic function, is transported bound to transferrin and enters cells via a transferrin receptor-mediated process. Cells that are in log phase growth demonstrate increased transferrin receptor density.[13] Thus, the increase in mammary [Tf-R] during pregnancy and early lactation could be a response to rapid cellular growth, stimulated by various growth factors, and the uptake of Tf.

It is possible that cellular needs at each stage of lactation dictate the requirement for diferric-transferrin. Iron is required for cell proliferation (DNA synthesis), collagen synthesis, and mitochondrial electron transport (energy production).[14] In early lactation, diferric-transferrin may be required for rapid cell proliferation, as well as for milk synthesis, so that on a concentration basis, there are more receptors expressed than in mid-lactation. One can speculate that during mammogenesis there is a greater need for transferrin for cell division than there is for the iron which is also internalized. Therefore, more iron is available for secretion resulting in a higher concentration of milk iron at day 2 of lactation. In addition, total milk production is low during this period.

The fact that the Tf-R content remained high beyond the initial growth phase presents an interesting question. If the primary growth phase is complete, why are transferrin receptors still expressed? Since epithelial cell ferritin levels are decreased, ruling out increased storage of iron, Shulman et al.[4] suggested that the continued presence of Tf-R may reflect a function involving the transport of iron into milk via transferrin produced within the cell. Our results showed that as lactation progressed beyond the initial growth phase, milk iron concentration decreased. However, there was no significant difference between each stage of lactation in the total milk iron content (Table 1) (based on calculations using values determined by Knight et al.[15] for daily milk yield in rats). Additionally, not all the iron found in rat milk is associated

Table 1 Changes in inguinal mammary gland weight and milk lactose concentration in lactating rats (day 2, day 14, and day 20 of lactation)

	Day 2	Day 14	Day 20	p value
Gland weight (g)	5.9 ± 0.2[a]	13.7 ± 0.8[b]	9.4 ± 0.7[c]	$p < .0001$
Mammary tissue as % body weight	2.0 ± 0.1[a]	4.2 ± 0.1[b]	3.5 ± 0.5[b]	$p < .001$
Lactose (%)	1.0 ± 0.1[a]	2.6 ± 0.5[b]	1.6 ± 0.1[ab]	$p < .01$
Total milk iron (μg) (estimated)	264 ± 54	225 ± 24	251 ± 49	NS

Numbers are expressed as means ± SEM with n = 5–7 animals per group.
Differences are determined by analysis of variance and are designated by different superscripts.

Table 2 Changes in relative concentration of transferrin receptor, total content of transferrin receptors and milk iron during lactation

	Day 2	Day 14	Day 20	p value
Relative [Tf-R]/μg protein	100%	65% (35% decrease)	54% (46% decrease)	<.001
Total Tf-R content	100%	268% (168% increase)	274% (174% increase)	<.0001
[Milk Fe] (μg/ml)	100%	38% (62% decrease)	49% (51% decrease)	<.001

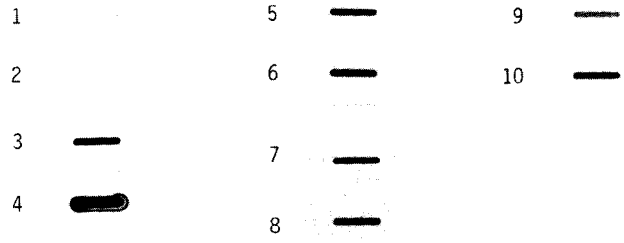

Figure 2 Representative slot blot of mammary tissue homogenates. Samples applied directly to nitrocellulose in pairs of 50 and 200 μl each.

Category	*Slot Number*
Non-lactating	1,2
Pregnant	3,4
Lactating, day 2	5,6
day 14	7,8
day 20	9,10

with transferrin; there is a significant portion of iron bound to the non-whey fractions of milk.[16]

Our interest in determining the relationship of the mammary Tf-R to milk iron stems from our concern for the high iron requirements of rapidly growing mammalian neonates and the relatively low levels of iron present in mammalian milk to meet these needs. The observations from this study suggest that it is the maternal need for iron for adequate mammogenesis and milk production, and not the growth needs of the neonate, which dictate the amount of iron present in mammalian milk. We hypothesize that iron may be required for milk production itself and/or for the oxidative phosphorylation cytochrome system as lactation is an energy expensive process. We have demonstrated that in rat dams fed a low iron diet throughout pregnancy and lactation, there is reduced milk production accompanied by reduced milk iron concentration.[17] At the same time, the amount of Tf-R present was the same as that for dams fed normal levels of dietary iron. Further work is needed to determine why milk iron levels decrease when there is an increase in total Tf-R in mammary tissue as well as to determine the extent of iron internalization into the mammary epithelial cell at different stages of lactation.

Acknowledgment

This research was partially funded by a pilot grant from the Clinical Nutrition Research Unit, University of California, Davis (grant #P30-AM235747); and by a USDA Graduate Student Fellowship.

References

1 Lönnerdal, B. (1986). Effect of maternal iron status on iron in human milk. In *Human Lactation 2: Maternal and Environmental Factors* (M. Hamosh and A.S. Goldman, eds.), p. 363–370, Plenum Press, New York

2 Sigman, M. and Lönnerdal, B. (1990). Characterization of transferrin receptors on plasma membranes of lactating rat mammary tissue. *J. Nutr. Biochem.* **1**, 239–243

3 Grigor, M.R., Wilde, C.J., and Flint, D.J. (1988). Transferrin receptor activity in rat mammary epithelial cells. *Biochem. Int.* **17**, 747–754

4 Shulman, H.M., Ponka, P., Wilezynska, A., Gauthier, Y., and Shyamala, G. (1989). Transferrin receptor and ferritin levels during murine mammary gland development. *Biochim. Biophys. Acta* **1010**, 1–6

5 Keen, C.L., Lönnerdal, B., Clegg, M., and Hurley, L.S. (1981). Developmental changes in composition of rat milk: trace elements, minerals, protein, carbohydrate and fat. *J. Nutr.* **111**, 226–230

6 Seligman, P.A. and Allen, R.H. (1987). Isolation of the trans-

ferrin receptor from human placenta. *Meth. Enzymol.* **147,** 239–247
7 Peterson, G.L. (1977). A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83,** 346–356
8 Clegg, M.S., Keen, C.L., Lönnerdal, B., and Hurley, L.S. (1981). Influence of ashing technique on the analysis of trace elements in animal samples. I. Wet ashing. *Biol. Trace Element Res.* **3,** 107–115
9 Dahlquist, A. (1964). Method for assay of intestinal disaccharidases. *Anal. Biochem.* **7,** 18–25
10 Hawkes, R., Niday, E., and Gordon, J. (1982). A dot-immunobinding assay for monoclonal and other antibodies. *Anal. Biochem.* **119,** 142–147
11 Mepham, T.B. (1987). In *Physiology of Lactation.* Open University Press, Philadelphia, PA, p. 112
12 Dembinski, T.C. and Shui, R.P.C. (1987). Growth factors and development and function. In *The Mammary Gland: Development, Regulation and Function* (M.S. Neville and C.W. Daniels, eds.), p. 355–381, Plenum Press, New York
13 Larrick, J.W. and Creswell, P. (1979). Modulation of cell surface iron transferrin receptors by cellular density and state of activation. *J. Supramolec. Struct.* **11,** 579–586
14 Vyas, D. and Chandra, R.K. (1984). Implications of iron deficiency. In *Iron Nutrition in Infancy and Childhood* (A. Stekel, ed.), p. 1–10, Raven Press, New York
15 Knight, C.H., Docherty, A.H., and Peaker, M. (1984). Milk yield in rats in relation to activity and size of the mammary secretory cell population. *J. Dairy Res.* **51,** 29–35
16 Loh, T.T. and Kaldor, I. (1972). Iron in rat milk: distribution between centrifugally separated phases. *J. Dairy Sci.* **57,** 399–440
17 Sigman, M. and Lönnerdal, B. (1990). Response of rat mammary gland transferrin receptors to maternal dietary iron during pregnancy and lactation. *Am. J. Clin. Nutr.* **52,** 446–450