Developmental Gene Expression of Lactoferrin and Effect of Dietary Iron on Gene Regulation of Lactoferrin in Mouse Mammary Gland*

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ABSTRACT

This study evaluated the developmental gene expression of lactoferrin (LF) and the effect of supplementary iron on gene expression of LF in the mammary gland of mice using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. In experiment 1, a total of 12 female mice were used to determine the effect of different lactating stages on mRNA expression of LF. The Institute of Cancer Research mice were divided into 4 groups; each group of 3 mice was tested on d 1, 9, 17, and 25 of lactation. In experiment 2, 6 groups of mice (total of 24 female mice at d 12 after mating) were fed purified diets (without iron or supplement iron) and were assigned to 2 treatments (control and treatment). The experimental feeding period lasted 35 d. During the feeding experiment, 6 mice (3 animals in each group) were chosen on d 1, 9, 17, and 25 of lactation to determine the effect of iron on LF mRNA expression of mice at different stages of lactation. The results of experiment 1 showed that LF mRNA had strong expression on d 1 of lactation, decreased gradually on d 9 and 17 of lactation, and then increased again markedly on d 25 of lactation. These results imply that the expression of LF in the mammary gland at different lactating stages is consistent with the changes in LF concentrations in milk. Iron significantly increased LF mRNA expression on d 1 and 25 of lactation. Iron did not statistically increase LF gene expression on d 9 and 17 of lactation. These findings raised the possibility that iron supplementation may play a role in regulation of LF levels in vivo.

(**Key words:** lactoferrin, mouse, gene expression, regulation)

Abbreviation key: LF = lactoferrin, RT-PCR = reverse transcription-PCR.

INTRODUCTION

Lactoferrin (LF), discovered originally in milk (Masson and Heremans, 1971), is a 78-kDa member of the transferrin family of iron-binding glycoproteins (Aisen and Listowsky, 1980; Metz Boutigue et al., 1984). Lactoferrin is expressed and secreted by glandular epithelial cells (Masson et al., 1966) and is found at lower levels in bodily excretion fluids such as tears, saliva, and mucosal and genital excretions, but it is found in high concentrations in milk of most species (Heremans and Schonke, 1969; Kussendrager, 1986; Yu and Chen, 1993). Mouse colostrum contains approximately 1 g/L of LF (Leclercq et al., 1987). Lactoferrin has been associated with a wide variety of biologically important processes, including regulation of iron metabolism (Iyer and Lonnerdal, 1993), protection against microbial infection (Weinberg, 1984), curbing the proliferation of fungi (Arnold et al., 1980) and viruses (Lu et al., 1987), cellular growth promotion (Hashizume et al., 1987), and regulation of immune function (Machnicki et al., 1993). Moreover, it has an extended role in the body's defense mechanism through its immune modulatory actions (Zucali et al., 1989).

The various functions of LF have received extensive attention, especially host defense and resistance against infections in mammary gland (Bullen et al., 1987; Tsui et al., 1990; Shimizu et al., 1996). The level of this protective action depends on the amount of LF in milk. In general, the amount of LF in milk varies with the different stages of lactation; it is high in colostrum and lower, by an order of magnitude, at full lactation (Klosba et al., 1976). It was reported that LF mRNA concentrations in bovine mammary tissue were quite low 2 d before parturition and during lactation but were high 3 d after the cessation of milking (Goodman and Schanbacher, 1991). Yet few reports have appeared in the literature concerning the levels of LF mRNA in mouse mammary gland tissues at different stages of lactation. Therefore, this study was conducted to measure the concentration of LF mRNA in samples of total mammary RNA prepared from mice at different stages of lactation. Specifically, the experiments were designed to determine whether the concentrations of

Received December 6, 2004.

Accepted January 25, 2005.

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*Supported by the National Basic Research Programme (2004CB117500) and Provincial Natural Science Foundation of Zhejiang (RC01053).

Table 1. Composition of purified diet.

Ingredient	Control	Treatment group
		(g/100 g)
Corn starch	39.0	39.0
Casein	26.0	26.0
Alpha-starch	10.0	10.0
Cellulose	5.0	5.0
Soybean oil	6.0	6.0
Sucrose	5.0	5.0
Dicalcium phosphate	5.0	5.0
Mineral mix 1 ^a	3.5	—
Mineral mix 2 ^b	—	3.5
Vitamin mix ^c	0.5	0.5

^aContained 56.8 g of MgSO₄·7H₂O, 14.1 g of NaCl, 26.4 g of KCl, 0.7 g of MnSO₄·H₂O, 0.1 g of CuSO₄·5H₂O, 0.4 g of ZnSO₄·7H₂O, 0.001 g of Na₂SeO₃, and 0.001 g of KI/100 g.

^bContained 56.8 g of MgSO₄·7H₂O, 14.1 g of NaCl, 26.4 g of KCl, 0.7 g of MnSO₄·H₂O, 0.1 g of CuSO₄·5H₂O, 0.4 g of ZnSO₄·7H₂O, 0.001 g of Na₂SeO₃, 0.001 g of KI and 16.1 g of FeSO₄·7H₂O (120 mg Fe/kg)/100 g.

^cContained 480,000 units of vitamin A, 51,400 units of vitamin D₃, 0.8 g of vitamin E acetate, 0.2 g of vitamin K₃, 0.4 g of vitamin B₁, 0.4 g of vitamin B₂, 0.4 g of vitamin B₆, 2.05 g of nicotinic acid, 0.8 g of Ca-pantoate, 0.2 g of folic acid, 7 mg of D-biotin, 0.8 mg of vitamin B₁₂, and 43 g of choline bitartrate/100 g.

mammary LF mRNA changed in a pattern to match the changes in milk LF concentrations.

In addition, the protein structure of LF has binding sites of Fe³⁺ ions. It contributes to the major structure-functional properties of LF. Some relevant genes (e.g., ferritin, transferrin receptor) have a specific 'iron regulator element' in the mRNA, and iron could up- or down-regulate these gene expressions (Klausner et al., 1993). However, no reports were found concerning the effect of dietary iron on gene expression of LF. The second aim of the present study was to investigate whether changes in iron supplementation might modify the mRNA concentration of LF in mammary gland of lactating mice.

MATERIALS AND METHODS

Animals and Purified Diets

Female ICR mice were purchased from the animal center at the Zhejiang College of Traditional Chinese Medicine (Hangzhou, Zhejiang, China) and were maintained and bred in the animal center. They were treated following the institutional guidelines for the care and use of experimental animals. The 24 female mice were divided into 2 groups at random at d 12 after mating, were kept in individual cages, and were provided purified diet with food and water ad libitum. The composition of purified diets (except iron) is listed in Table 1. The difference was 0 mg/kg of Fe in the control diet and 120 mg/kg of Fe in the treatment diet.

Tissue

Mouse mammary gland samples were prepared from the female ICR mouse on d 1, 9, 17, and 25 after parturition. At the indicated time, mice were killed by decapitation, and mammary gland tissues were aseptically removed and immediately frozen in liquid nitrogen for RNA isolation.

Total RNA Extraction

Total RNA was isolated from the maternal mammary gland tissues (entire mammary gland pulverized for the analysis) at the indicated time using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacture's manual. After pulverization and homogenization of the tissue, the homogenate was extracted with chloroform and then precipitated by isopropanol. The resulting pellets of total RNA were dissolved in ultra pure water; the quantity and the quality of total RNA were measured by a spectrophotometer at 260 and 280 nm.

Reverse Transcription

Two micrograms of total RNA were converted into cDNA; 2 µg of total RNA and 2 µL of random primers (500 µg/mL; Promega Corp., Madison, WI) were denatured at 70°C for 5 min. The following components were added to give a total reaction volume of 25 µL: 5 µL M-MLV 5× reaction buffer (250 mM Tris·HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂, and 50 mM dithiothreitol), 2 µL dNTP mix (10 mM each of dATP, dCTP, dGTP, and dTTP), and 1 µL M-MLV reverse transcriptase [200 units/µL; 0.5 µL rRNasin ribonuclease inhibitor and nuclease-free water (Promega Corp.)]. It was mixed gently by flicking the tube, and the reaction mixture was incubated at 37°C for 60 min.

Determination of the Number of PCR Cycles

It is important to select the appropriate number of cycles so that the amplification product is not only clearly visible on an agarose gel and can be quantified, but is also in the exponential range and does not reach a plateau yet. One microliter of cDNA solution, obtained by reverse transcriptase reaction of total RNA, was used for templates of PCR amplification in a total volume of 50 µL. The optimum PCR primer concentration, Mg²⁺ concentration and annealing temperature to give a linear amplification of each transcript were determined by a preliminary experiment (data not shown). The PCR assay mixture contained the following components: 37.5 µL nuclease-free water, 5 µL 10× PCR reaction buffer, 3 µL MgCl₂ (25 µM), 1 µL dNTP mix, 1 µL

Table 2. Specific primers of lactoferrin and β -actin gene.

Gene	Oligonucleotide sequence	PCR product (bp)
Lactoferrin	5'- GCTGGAGATGTGGCTTTTAC -3'(sense primer)	897
	5'- CAGAGATTGGATTGGGGTC -3'(antisense primer)	
β -actin	5'- CGGGACCTGACCGACTACCT -3'(sense primer)	411
	5'- GGCCGTGATCTCCTTCTGC -3'(antisense primer)	

sense primer (20 μ M), 1 μ L antisense primer (20 μ M), and 0.5 μ L *Taq* DNA polymerase (2 U/ μ L; Promega Corp.). All subsequent amplification reaction steps were performed using a Gene Amp PCR system 9600. The PCR profile for murine LF included denaturation at 94°C for 2 min, followed by different cycles of denaturation at 94°C for 50 s, annealing at 55°C for 50 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR profile for β -actin was similar to that of LF, except that annealing was done at 58°C. Oligonucleotide primers specific for murine LF and β -actin are based on known sequences deposited in Genebank and listed in Table 2. The PCR amplification products from mRNA were predicted to be 897 and 411 bp for LF and β -actin, respectively. The oligonucleotide primers used had been synthesized by Shanghai Sangon Biological Engineering Technology and Service Co. (Shanghai, China). The LF and β -actin PCR cycles were performed 23, 25, 27, 29, 31, 33, and 35 times, respectively, when the other PCR parameters were consistent. Then, the appropriate cycle numbers were confirmed.

Reverse Transcription-PCR Assay

The relative concentration of LF mRNA in different lactating stages and different groups were determined by the semi-quantitative reverse transcription-PCR (RT-PCR) method (Marone et al., 2001). An equal volume of one of the same sample cDNA was used for LF and β -actin PCR, respectively, as described previously. The component of PCR was the same as that described previously, and the incubation cycle was 29 cycles for LF and β -actin amplification.

A 5- μ L portion of each PCR product was subjected to electrophoresis on a 1.0% agarose gel with ethidium bromide. The PCR products were normalized according to the amount of β -actin detected in the same cDNA sample, and LF/ β -actin ratios were calculated. The expression level of LF gene in mouse mammary gland at different lactation stages and different groups were compared on the basis of LF-to- β -actin ratio.

Data Analysis

Electrophoresis band intensities of the PCR products were quantified using Image Master VDS software

(Amersham Pharmacia Biotech, Uppsala, Sweden). Mean LF mRNA expression levels normalized against β -actin levels from mammary gland tissue were presented in absolute integrated optical density. Each value was analyzed for statistical difference according to the Bonferroni/Dunn method. Differences between groups were analyzed by the Student *t*-test when 2 groups were analyzed, and ANOVA (Duncan, 1955) was used when more than 2 groups were analyzed.

RESULTS

Number of PCR Cycles

After amplification of mammary gland cDNA using specific primer for mouse LF and β -actin, PCR products were observed at 897 and 411 bp, respectively (Figure 1A, B). As shown in Figure 1, the band intensity of electrophoresis on a 1.0% agarose gel with ethidium bromide showed stronger positive signals by the increased cycle. Then, for the semi-quantification analysis, the amplification cycle was used for 29 cycles for LF and β -actin. Twenty-nine cycles of amplification were sufficient to allow the visualization of the LF messenger in the mouse lactating mammary gland, and it did not reach a plateau.

LF Gene Expression in the Mammary Gland of Lactating Mice

Reverse transcription-PCR was conducted on the total RNA extracted at 4 stages of lactating development (d 1, 9, 17, and 25 of lactation) from mouse mammary gland tissues. The constitutively expressed housekeeping gene β -actin was used as an internal control. Using primers for LF and β -actin, 2 bands were obtained from mouse mammary gland (897 and 411 bp) (Figure. 2).

Figure 2 displays the bands of mice LF and β -actin PCR product that were subjected to electrophoresis on a 1.0% agarose gel with ethidium bromide. The integrated optical density of LF and β -actin bands of mice was determined (Figure 3). The levels of LF mRNA in the mammary gland tissues were measured and corrected by that of β -actin mRNA, and the corrected values were used as an index of LF gene expression (Figure 3).

Figure 1. A) Electrophoresis result of lactoferrin and β -actin PCR products collected from cycles 23, 25, 27, 29, 31, 33, and 35 of mouse mammary gland tissue on a 1.0% agarose gel. B) Integrated optical density analysis of lactoferrin and β -actin PCR products at 23, 25, 27, 29, 31, 33, and 35 cycles. The bands were densitometrically quantified by Image master VDS.

Reverse transcription-PCR analysis showed that the levels of LF mRNA in mice mammary gland tissues at different lactating stages varied in a biphasic manner. Lactoferrin mRNA levels decreased steadily between d 1 after parturition and d 9 after parturition and were significantly lower at d 9 after parturition ($P < 0.05$). However, at d 17 after parturition, the level of LF mRNA also remained significantly lower than at d 1 after parturition ($P < 0.05$). But, no significant differences were detected in LF expression at d 17 after parturition compared with d 9 after parturition. Thereafter, LF gene expression increased significantly along with the involution of mammary gland tissue. The LF gene expression at d 25 after parturition was markedly higher than at d 9 and 17 after parturition ($P < 0.05$). The gene expression of LF at d 25 after parturition was not significantly higher than at d 1 after parturition (Figure 3).

Effect of Iron Supplementation on Gene Expression of LF

The effect of dietary iron on mRNA concentration of LF in mammary gland tissues at different lactating

stages was examined, and the results, presented in Figure 4, show that iron has an effect on LF gene expression and that supplemental iron improved the levels of LF mRNA at 4 stages after parturition. However, the increased levels of LF gene expression at these 4 stages are different. Compared with the control group, the increase in LF gene expression in mammary gland tissues in the treatment group was not significant at d 9 and 17 of lactation. But, at d 1 and 25 after parturition, the expression of LF in the mammary gland increased greatly in response to supplemental iron ($P < 0.05$). The integrated optical density ratios of LF to β -actin in mice on the control and treatment diets at different lactating stages are shown in Figure 5.

DISCUSSION

The mammary gland tissue has long been recognized as the major site of LF expression in the mouse and other animals (Groves, 1960). In the current experiment, developmental changes in LF mRNA levels at d 1, 9, 17, and 25 after parturition in mammary gland tissues of female mice were evaluated by semi-quantitative PCR analysis. Reverse transcription PCR allows

Figure 2. Developmental gene expression of lactoferrin in mammary gland tissue during lactation of mice was analyzed by RT-PCR. Amplified PCR products obtained with mRNA after reverse transcription were size-fractionated on a 1.0% agarose gel. The gel was stained with ethidium bromide, and PCR products were observed under UV light (Image master VDS). Showing at the images were bands of the expected sizes of RT-PCR products of mouse lactoferrin ($\times 29$ PCR cycles) and β -actin ($\times 29$ PCR cycles), which was used as an internal control. Lanes 1 through 4 = amplified PCR products of lactating mouse mammary gland tissue at d 1, 9, 17, and 25 of lactation, respectively. Three repetitions are represented by a, b, and c.

Figure 3. The integrated optical density (IOD) ratio of lactoferrin and β -actin electrophoresis bands at different lactating stages. Densitometric analysis of mice lactoferrin was normalized to β -actin and is shown as lactoferrin-to- β -actin ratios. Cumulative results (mean $\pm$ SEM) are shown in a histogram. The results are representative of 3 independent experiments using 3 mice. * $P < 0.05$.

the relative quantification of LF mRNA levels of mammary gland tissues, although not the absolute quantification. The resulting 897-bp amplification has been previously sequenced from mouse mammary gland and was 99% homologous to the known sequences of LF deposited in Genbank.

The present data clearly demonstrated that there are some variations in levels of mRNA encoding LF in mammary gland tissues, and the differences in LF expression were stage-dependent. Lactoferrin mRNA has strong expression at d 1 after parturition. Then, the amount decreased steadily at d 9 and 17 after parturition. There are reports that the concentration of LF in milk varies with the different stages of lactation (i.e., it is high in colostrum and lower, by an order of magnitude, at full lactation) (Neville et al., 1998; Tsui et al., 1990). The results from the current experiment imply that the expression amount of LF in the mammary gland at different lactating stages is consistent with the changes in LF concentrations in milk. Research has suggested that LF is a major gene product in estrogen-stimulated mouse uterine tissue (Pentecost and Teng, 1987; Liu and Teng, 1992), and its gene expression was shown to be regulated by estrogen (Pentecost and Teng, 1987; Newbold et al., 1992). However, the regulation of estrogen to LF gene expression is tissue-specific. The synthesis of LF in mouse mammary gland is unaffected by estrogen, but is dependent on prolactin (Green and Pastewka, 1978; Liu and Teng, 1992).

In the current study, stronger LF mRNA expression was observed (Figure 2) during the later involution period of the mammary gland (at d 25 after parturition). At this time, it was likely to have other potential factors to regulate the expression of LF mRNA, except for prolactin. The result from Lee et al. (1996) also indicated that the stronger expression of LF was observed during mammary gland involution. The increasing of LF levels may contribute to the prevention of microbial infection

Figure 4. Effect of dietary iron on mammary gland tissue lactoferrin gene expression during lactation of mice was analyzed by RT-PCR. Amplified PCR products obtained with mRNA after reverse transcription were size-fractionated on a 1.0% agarose gel. The gel was stained with ethidium bromide, and PCR products were observed under UV light (Image master VDS). Shown are the images of bands of the expected sizes of RT-PCR products of mouse lactoferrin ($\times 29$ PCR cycles) and β -actin ($\times 29$ PCR cycles), which was used as an internal control. Lane 1 = 0 mg Fe/kg; lane 2 = 120 mg Fe/kg. A, B, C, and D were amplified PCR products of lactating mouse mammary gland tissue at d 1, 9, 17, and 25 of lactation, respectively. The results are representative of 3 independent experiments using 3 mice.

Figure 5. The integrated optical density (IOD) ratio of each band of lactoferrin and β -actin of control and treatment mice at different lactating stage. Densitometric analysis of control and treatment mice lactoferrin were normalized to β -actin and is shown as lactoferrin-to- β -actin ratios. Each column is the mean of 3 individual means ($\pm$ SEM) * $P < 0.05$.

during mammary involution, at which stage the residual milk in the mammary gland provides good nutritional sources for microbial growth (Sanchez et al., 1992). Different regulation mechanisms of tissue and stage-specific LF gene expression should be studied in the future.

We further investigated the effect of dietary iron on gene expression of LF in vivo by semi-quantitative RT-PCR. Our results showed that iron increased LF mRNA levels in the mammary gland of female mice during lactation. The levels of LF mRNA in the treatment group (120 mg of Fe/kg) were higher than in the control group (0 mg of Fe/kg) at the 4 stages. The result of this study has some consistency with the review of Wang et al. (2002). Wang et al. (2002) investigated the effect of Fe^{3+} (100 to 1000 μM of FeCl_3) on the expression of rPLF (recombinant porcine LF) in a *Pichia pastoris* transformant in shake-flask cultures. Results revealed that addition of Fe^{3+} to 100 μM significantly enhanced the expression of rPLF without interfering with cell growth (Wang et al., 2002).

As a member of the transferrin gene family (Park et al., 1985), the iron-binding site and the overall struc-

ture of LF are rather similar to those of the plasma iron-transport protein transferrin (Baker et al., 2002), but LF binds iron more tightly than transferrin (Bennett et al., 1981). The regulation of transferrin protein levels in the rats by iron has been well documented (Idzerda et al., 1986; Grigor et al., 1990). It was reported that the influence of iron on transferrin gene expression has connection with transferrin receptor. The biosynthesis of transferrin receptor can be regulated post-transcriptionally by iron in a common mechanism. It has been suggested that the number of transferrin receptors in the mammary gland will be up- or down-regulated during iron deficiency or sufficiency, because it has a specific "iron regulator element" in its mRNA (Sigman and Lönnerdal, 1990).

Our results have also shown that the influence of dietary iron on LF gene expression was different at 4 stages of lactation. A marked increase in the expression of LF mRNA might occur at d 1 and 25 after parturition. However, the influence of iron was not significant on the expression of LF mRNA at d 9 and 17 after parturition. It is possible that some regulation factors of LF gene expression were more sensitive at special physiology conditions (e.g., 1 d after parturition, involution period of mammary gland).

It is well known that iron is an essential element for normal body function, and it can affect a host of cell functions, such as gene regulation, DNA, and protein biosynthesis (Machnicki, 1991). The potential importance of iron to the gene can be appreciated from the facts that control of iron consumption occurs by down-regulating the expression of iron-containing proteins under iron-restricted conditions (Andrews et al., 2003). It seems likely that such iron-dependent regulation of biosynthesis mediated by the iron-responsive element binding protein also occurs in LF receptors that existed in intestinal epithelial cells, which may be regulated in response to intracellular iron levels. Thus, the LF receptor may play a role in iron absorption (Hu et al., 1988). Further studies are needed to document whether LF receptor is present in the mammary gland of the mouse. If this is true, our present study suggests that the regulatory mechanism of iron to LF is realized by the effect of iron on LF receptor expression.

This study showed that LF mRNA concentration is high at the beginning and end of the lactation and low between these points and that LF mRNA concentrations can be increased by the addition of supplementary iron to the diet.

ACKNOWLEDGMENTS

This study was supported in part by the animal center at the College of Zhejiang Traditional Chinese Medi-

cine, and we are grateful to the help of Director Minli Chen in the early part of this work.

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