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Adenoviral Vector Mediates High Expression Levels of Human Lactoferrin in the Milk of Rabbits

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The limitations in current technology for generating transgenic animals, such as the time and the expense, hampered its extensive use in recombinant protein production for therapeutic purpose. In this report, we present a simple and less expensive alternative by directly infusing a recombinant adenovirus vector carrying human lactoferrin cDNA into rabbit mammary glands. The milk serum was collected from the infected mammary gland 48 h post-infection and subjected to a 10% SDS-PAGE and Western blotting. An 80-kDa protein was visualized after viral vector infection. With this method, we obtained a high level of expressed human lactoferrin of up to 2.3 mg/ml in the milk. Taken together, the method is useful for the transient high-level expression recombinant proteins, and the approach established here is probably one of the most economical and efficient ways for large-scale production of recombinant proteins of biopharmaceutical interest.

Keywords: Adenovirus vector, human lactoferrin, mammary gland, milk, rabbit

Human lactoferrin (hLF) is an iron-binding glycoprotein that is strategically situated at the mucosa and is considered as an important component of the nonspecific immune system [14, 17, 31]. This protein is produced by epithelial cells and is found in mucosal secretions such as tears, saliva, nasal exudates, gastrointestinal fluids, and seminal and vaginal fluids [21]. The primary structure of hLF consists of 692 amino acids in its mature protein with a molecular mass of 80 kDa. It contains two homologous globular lobes, each with one iron-binding site [14]. Previous studies suggested that hLF has many important physiological functions. For example, hLF has been demonstrated to have antibacterial [2, 4, 10, 15, 20], antiendotoxin [35], and antiviral [30] activities. In addition, many other functions have been proposed for hLF, including promotion of lymphocyte growth [7, 18], stimulating production of cytokines [5], and modulating the inflammatory process and innate host defense [21].

Given the nutritional and therapeutic applications of hLF, it is urgent to design efficient expression systems for production of functional recombinant hLF. Until now, many attempts have been made to produce recombinant hLF for structure and functions studies but were not suitable for a large-scale production [13, 15, 19, 25, 26, 33]. Recently, there was a report on attempts to use the transgenic cattle for a large-scale production of hLF [29]. However, owing to its technique difficulties and cost, transgenic animals have not been considered as a practical approach to produce recombinant proteins on a large scale.

The problems mentioned above have stimulated the development of an efficient and economical expression system for production of hLF. The direct *in vivo* infection of the mammary gland with recombinant adenoviruses was proposed as an effective and versatile alternative to target the expression of exogenous genes to the secretory mammary epithelial cells [23]. Although many investigations have been made to employ this approach to produce the target protein [6, 11, 24, 27, 28, 32, 34], only a few reports have successfully obtained the recombinant protein in the milk of animals [6, 27, 28].

In the present study, we investigated this alternative approach to express hLF *via* direct *in vivo* infection of secretory mammary epithelium with recombinant adenovirus carrying the hLF cDNA. We successfully expressed hLF in rabbit milk and obtained the highest expression level of the

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recombinant protein, up to more than 2.3 mg/ml. Furthermore, the recombinant protein expression period lasted for more than 10 days.

Materials and Methods

Cell Lines, Medium, and Culture Maintenance

Human embryonic kidney (HEK) 293 cells were obtained from Microbix Biosystems Inc. and grown in minimal essential medium (MEM), supplemented with 10% (v/v) fetal bovine serum, 1% nonessential amino acid, 2 mM L-glutamate, and 1% penicillin-streptomycin.

Primary rabbit mammary epithelial (RME) cells were isolated from early-lactation rabbits as described previously [1]. Some modifications have been made as follows. Rabbit mammary parenchymal tissue was finely minced with scissors and digested overnight in a shaker incubator at 37°C in MEM supplemented with collagenase. After addition of DNase, the digestion flask was returned to the incubator for 15 min. The tissue suspension was then centrifuged at 200 ×*g* for 5 min at room temperature. The cell pellet was resuspended in MEM. This procedure was repeated three times. The final cell suspensions were passed through a 53-μm-pore size filter with epithelial cells remaining on the filter. The RME cells were finally removed from the filter and maintained in MEM supplemented with 10% FBS, epidermal growth factor (10 ng/ml), insulin (10 μg/ml), and 1% penicillin-streptomycin.

hLF cDNA Amplification and Cloning

The full length of the hLF cDNA was directly amplified by one-step PCR from a human brain cDNA library. The primers used in the PCR amplification were designed as follows: forward primer, 5'-

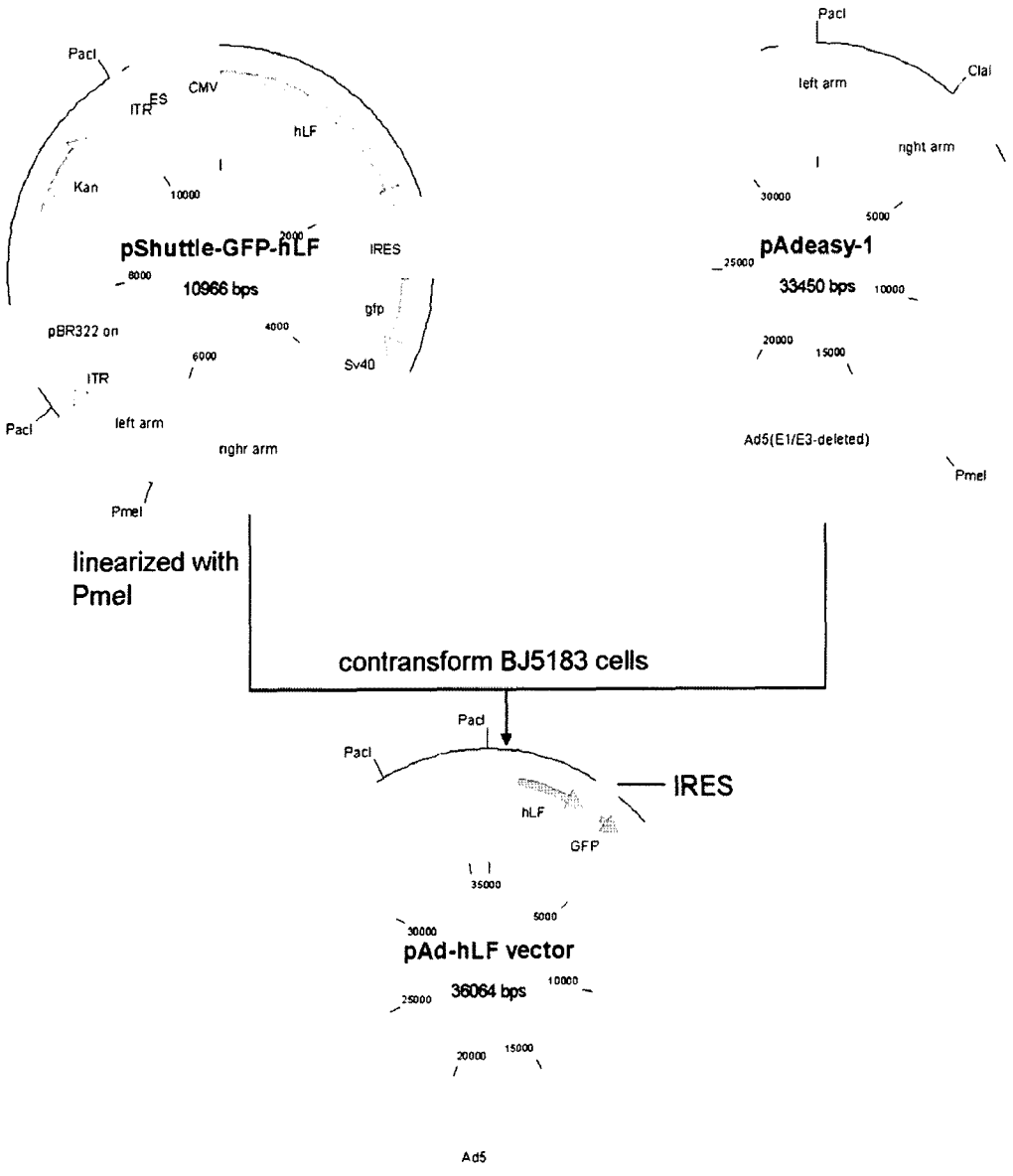

Fig. 1. Construction of recombinant adenoviral vectors containing the human lactoferrin cDNA gene.

tcggtaccgacatgaaacttgtcttcc-3'; reverse primer, 5'-cgctcgagttacttcctgaggaattc-3'. To facilitate downstream subcloning of PCR products, we added KpnI and XhoI (underlined) sites into the 5' ends of the primers, respectively. PCR was carried out under the following conditions: started at 94°C for 5 min, followed by 30 cycles of a denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 2 min 30 s, and ended at 72°C for 6 min. PCR products were cloned and sequenced.

Construction of Recombinant Adenovirus Vector and Virus Preparation

To monitor the recombinant hLF gene expression *in vitro* and adenovirus infection efficiency *in vivo*, we employed the internal ribosome entrance site (IRES) system to express hLF and green fluorescent protein (GFP) from the same promoter CMV. To construct this expression vector, we first designed two primers (forward: 5'-tttaagcttaccatggtgagcaagggcgag-3'; reverse: 5'-cccgatatcttacttgtacagctcgtccatg-3') to amplify the 735-bp GFP gene from the pEGFP-N1 vector (TaKaRa). The PCR product was digested with HindIII and EcoRV enzymes. Following gel purification, the PCR fragment was cloned into the corresponding sites of the vector pcDNA3.1(+) (Invitrogen). The resulted plasmid, named as p3Lf, was then digested with NotI and PmeI, and the 753-bp fragment containing the complete sequences for GFP was cloned into the plasmid pIRES (TaKaRa), designed as pIR-G. The NheI and XhoI fragments containing the hLF cDNA from the plasmid p3Lf was finally cloned into the corresponding sites of the pIR-G plasmid. The resulted plasmid was designated as pLf-GFP.

The replication defective adenovirus vector pAd-hLF was generated as described previously [8]. In brief, the pShuttle-CMV vector (Stratagene) was first digested with BglII and blunt ended with Klenow polymerase (Promega), and then digested with NotI. The 3,544-bp PmeI/NotI fragment containing hLF and GFP genes from the plasmid pLf-GFP was cloned into the pShuttle-CMV vector (Stratagene). The resulted vector was designated as pShuttle-GFP-hLF. The AdEasy vector (Stratagene) containing the adenovirus type 5 genome deleted for E1 and E3 regions was first transformed into the ultral-competent BJ5183 bacteria to obtain the AdEasy bacteria. The plasmid pShuttle-GFP-hLF was then transformed to the AdEasy bacteria, yielding the pAd-hLF recombinant adenovirus vector (see Fig. 1). The pAd-hLF vector was linearized with PacI and purified by the commercial purification kits (Qiagen) according to the manufacturer's instructions.

The viral stocks were produced by transient transfection of HEK 293 cells using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To amplify further, the adenovirus stocks were diluted appropriately and added to thirty T75 flasks. The recombinant adenovirus was harvested 48 h post-infection and the cell pellets from all flasks were combined. The final cells pellet was resuspended in 5 ml of sterile phosphate-buffered saline (PBS). The cells were lysed by four cycles of freezing/thawing. Cell lysate was centrifuged at 2,500 ×g for 5 min at 4°C. The titer of the recombinant adenovirus preparation was determined by GFP expression on semiconfluent HEK 293 cells. The final virus stock was stored at -70°C until use.

Animals

Adult female rabbits (13±0.5 kg) were bred from a local rabbit farm and kept in the animal facility at the School of Biological Engineering, University of Yanshan. All animal procedures were approved by the Animal Care and Use Committee of the University of Yanshan.

***In Vitro* Expression Assay on RME Cells**

The RME cells prepared as described above were seeded in 100-mm dishes at a density of about 2×10^5 cells/cm^2 in MEM supplemented with 10% FBS, epidermal growth factor (10 ng/ml), insulin (10 μg/ml), and 1% penicillin-streptomycin. When cell density reached up to 70% confluence, the medium was replaced with fresh MEM without serum. The cells were infected with the recombinant adenovirus pAd-hLF at multiplicity of infection (MOI) of 5, 25, 50, and 100, respectively. Seventy-two hours post-infection, cells and medium were harvested to determine hLF expression.

Infusion of hLF Recombinant Adenoviruses into Rabbit Mammary Glands

Rabbits were anesthetized for adenovirus infusion. For all mammary infusions and sample collections, teats were routinely wiped with 70% alcohol and iodine to prevent mammary gland infection. The recombinant adenoviruses were infused directly into the left mammary glands of each rabbit. The left ventral mammary glands of each rabbit were infused with 200 μl of a solution of PBS containing the desired amount of the recombinant adenoviruses by using a 1-ml syringe. As an intra-animal control, the right mammary glands received the same volume of sterile PBS.

The treated rabbits were milked on the indicated days. Milk samples from the same experimental group were pooled for analysis. The milk was collected and immediately centrifuged at 10,000 ×*g* for 20 min at 4°C. The supernatant was separated from the layer as completely as possible, and then recentrifuged at 10,000 ×*g* for 20 min at 4°C. The milk serum was stored in aliquots at -70°C for later use.

Western Blot Analysis of the hLF

Protein samples were mixed with equal volumes of loading buffer (10% β-mercaptoethanol, 0.2% SDS), followed by boiling samples for 5 min. After separation in 10% SDS-PAGE, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore) using a semi-dry transfer method. The membrane was blocked with 5% nonfat dried milk in PBS with 0.05% Tween-20 (PBST) for 1 h at room temperature, washed two times with PBST, and incubated with PBST containing 5% of nonfat dried milk and mouse anti-human lactoferrin monoclonal antibody for 1 h at 37°C. The membrane was then washed three times (each for 10 min) with PBST followed by a 1 h incubation with horseradish-peroxidase-labeled goat anti-mouse IgG antibody at room temperature. After four times of washing with PBST, the hLF was detected by using the ECL kit (Amersham).

Determination of the hLF by ELISA

Concentration of the recombinant hLF in the milk serum sample was determined by a commercial human lactoferrin ELISA kit (USBiological) following the manufacturer's instructions. The ELISA standard curve was prepared by using a serial dilution of the hLF standard protein from 1.6 to 100 ng/ml. The absorbance at 450 nm was measured using a microplate reader. The concentration of hLF was calculated from the OD_{450} values based on the hLF ELISA standard curve.

Results

Transduction of Rabbit Mammary Epithelial Cells *In Vitro*

To simplify the titration of recombinant adenoviruses and to monitor the recombinant hLF gene expression, we constructed an adenoviral vector containing an internal ribosome entry site (IRES) sequence derived from the encephalomyocarditis virus (ECMV). The GFP and hLF genes were respectively cloned into the downstream and upstream of the IRES region under the control of a CMV promoter. The incorporation of the IRES sequence and GFP encoding sequence in this vector was designed to serve as a live marker for examining infected cells or tissues in animal studies.

To determine the ability of the pAd-hLF vector to transduce RME cells, the fresh isolated primary rabbit mammary epithelial cells were infected with MOI of 5, 25, 50, and 100, respectively, and transduction efficiency was assessed by examination of GFP expression. Twenty-four hours after the infection, the transduction effect of primary RME cells by the pAd-hLF vector was detected through GFP expression in almost 100% of the cells for all the MOI treatments (data not shown). The intensity of fluorescence, however, was dependent on the numbers of the viruses infected in the experiment. This high efficient transduction of primary RME cells could be related to abundant expression of the coxsackievirus and the adenovirus receptor (CAR) in the RME cells, which suggested that the same phenomenon is likely to happen *in vivo* in the rabbit's mammary gland.

Since the GFP gene was cloned downstream of the IRES sequence controlled by the same CMV promoter, the intense GFP fluorescence indicated the high expression level of hLF protein. Indeed, the high efficient transduction of primary RME cells resulted in high expression levels of hLF in the culture medium (see Fig. 2). Western blot analysis indicated that the hLF gene was expressed as a protein of about 80 kDa. In contrast, no recombinant hLF protein was detected in medium collected from the mock-infected primary RME cells (see Fig. 2A). Although the hLF expression level in primary RME cells was adenoviral dose-dependent, a nonlinear relationship was found between the adenovirus load and the amount of hLF secreted in the medium (see Fig. 2B). ELISA analysis demonstrated that the hLF expression level increased from an initial level of 58 μg/ml at a MOI of 5 to 520 μg/ml at a MOI of 25. However, when the adenovirus load was increased to MOI of 50 and 125, hLF expression levels dropped to 496 μg/ml and 390 μg/ml, respectively. This fall in the expression levels seems to be the result of the high cell death observed during the cell harvest. Cell death, apparently associated with the cytopathic effect caused by the high viral dose, suggests the existence of an optimal viral dose over which cell damage precludes the expression of the foreign protein. Therefore, the MOI of 25 seems to be the best dosage for the further study.

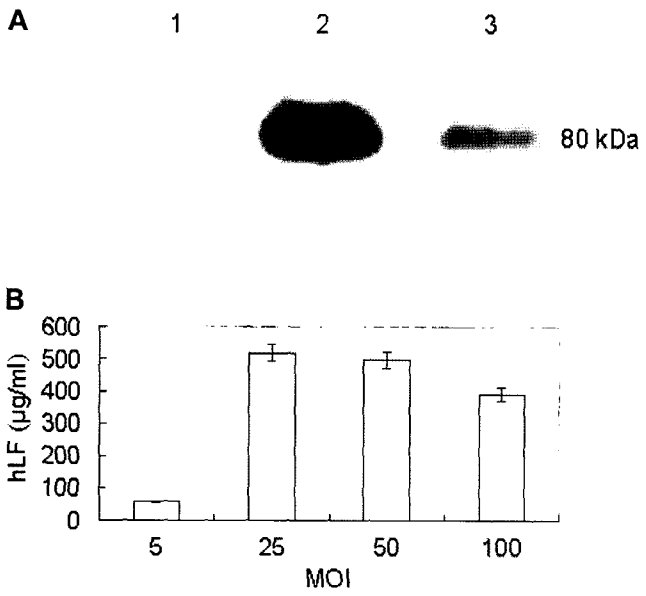

Fig. 2. Transient infection of RME cells by using a recombinant adenovirus vector carrying hLF cDNA.
A. Western blotting analysis of Ad-hLF-infected RME cells. Ten μl of medium samples was loaded in each well; the total protein contents were 1 μg. Lane 1, control; lane 2, Ad-hLF-vector-infected cells; lane 3, Standard protein of hLF (0.5 μg). **B.** ELISA determination of hLF expression in RME cells infected with different MOIs.

Transduction of Rabbit Mammary Gland *In Vivo*

Determination of the Optimal Time for Adenovirus Infusion. As previous studies suggested that the physiological stage of animal mammary glands plays an important role in efficient adenovirus infection, we first designed five experimental groups to determine the optimal time for adenovirus administration. The ventral mammary glands (MG) of rabbits were infused with 1×10^8 PFU per MG on days 1 and 10 postpartum and days 27, 28, and 29 of gestation, respectively. Each group consisted of at least

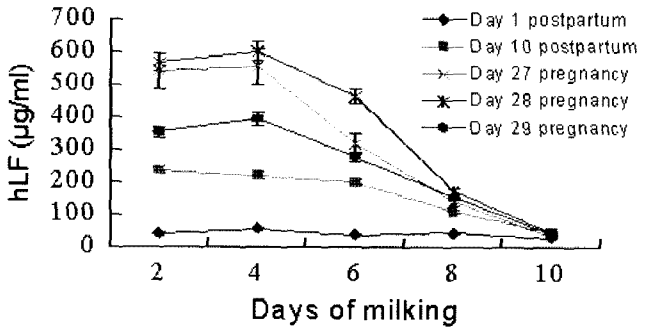

Fig. 3. Expression of hLF in rabbit mammary glands transfected at different developmental stages with Ad-hLF vector.
Error bars, Standard deviations calculated from the data (n=3).

two female rabbits. Milk was collected on days 2, 4, 6, 8, and 10 post-infusion when lactation started. At the same time, uninfected mammary glands were milked at the indicated days as a negative control.

The hLF protein in milk was detected in all five experimental groups by Western blot analysis, but not detected in the milk from uninfected mammary glands (see Fig. 3). We further found that the higher expression levels of hLF were obtained from the groups infected with the recombinant adenoviruses at late pregnancy ($P<0.05$). Among these late pregnancy groups, the rabbits infused on day 28 of pregnancy yielded the hLF at the level of 568 μg/ml on the second day of lactation, whereas the animals infused with the recombinant adenoviruses on day 1 postpartum produced the lowest hLF (less than 60 μg/ml). The low expression levels might be the result of either the presence of residual milk in the mammary gland that could interfere with the recombinant adenoviruses reaching to distal regions of the mammary gland or due to the fact that the epithelium is turning over rapidly at the postpartum stage.

Further examination of hLF recombinant protein during the late lactation indicated that the hLF expression decreased gradually as lactation continued (see Fig. 3). Although the expression level of hLF in the group infused on day 28 pregnancy seemed to be slightly higher than that obtained in the groups infused on day 27 pregnancy, there was no significant difference observed between these two treatment groups ($P>0.05$). Therefore, we considered the adenovirus infusion on day 28 pregnancy of rabbits as the optimal time and used it for further experiments.

Correlation of the Adenoviral Vector Dose with hLF Expression Levels

To achieve the highest hLF recombinant protein production from the milk of rabbits, we next examined the effect of adenovirus dose on hLF expression. Four different virus loads were designed to infuse the ventral mammary glands of rabbits on day 28 gestations with 1×10^7, 2×10^8, 4×10^8, and 1×10^9 PFU/MG, respectively. Following adenovirus administration, the milk was collected daily from day 1 until day 10 of lactation. Uninfected mammary glands were also milked on the indicated days and used as an intra-animal negative control. The recombinant human lactoferrin was detected in the milk by Western blot in all four adenoviral doses used (see Fig. 4A). Western blot analysis indicated that the hLF recombinant protein expressed in rabbit milk showed a very similar migration with the commercial hLF available from Sigma. During the course of the experiments, none of the rabbits used in this study showed symptoms of mastitis.

As shown in Fig. 4B, the adenovirus doses used in infusing mammary glands significantly affected the amount of hLF produced in infected rabbit milk. Rabbits infused with 4×10^8 PFU/MG produced the hLF in the milk at an

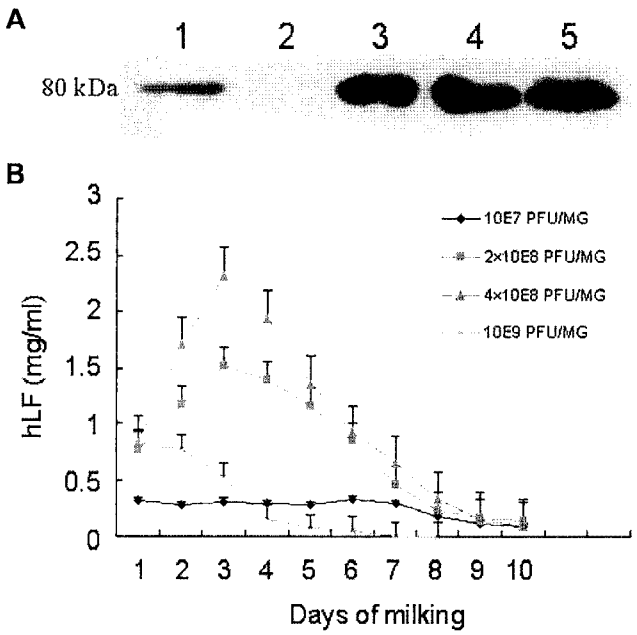

Fig. 4. Temporal secretion of hLF in transfected mammary glands of rabbits.

A. Western blotting analysis of milk serum in rabbit infused with adenoviral vector Ad-hLF. Five μl of milk serum samples was loaded in each well. Lane 1, Standard hLF protein (Sigma); lane 2, milk serum samples from uninfected mammary gland; lanes 3–5, milk serum samples from infected mammary glands of rabbits during lactation days 3 to 5. **B.** Expression of hLF in rabbit mammary glands transfected at different dosages with Ad-hLF vector.

average of 1.0 mg/ml, whereas the groups infused with 1×10^7 PFU/MG, 2×10^8 PFU/MG, and 1×10^9 PFU/MG expressed at an average 0.24 mg/ml, 0.78 mg/ml, and 0.39 mg/ml, respectively. In rabbits infused with 4×10^8 PFU/MG, the highest hLF expression level of 2.3 mg/ml was obtained at day 3 of milking. This was about eight times higher than that obtained on the same day in rabbits infused with 1×10^7 PFU/MG, which showed that higher virus dose resulted in higher hLF expression. However, further increase of adenovirus dose up to 1×10^9 PFU/MG did not improve the expression level of hLF. The highest expression level of hLF in this group was 0.81 mg/ml on day 1 of milking and after that, the expression levels of hLF dropped drastically between days 4 and 6 of lactation. This fall seemed to be a result of mass of death of the infected epithelium due to the cytopathic effect associated with the adenovirus infection or due to the strong cytotoxic response of the host immune system against the infected cells. These results suggested that there exists a limit to the amount of adenoviruses infused, and also related to the yields of the secreted recombinant protein. Taking these factors into consideration, it is possible that the use of high-capacity "gutless" adenovirus vectors seems to be a promising alternative. These vectors can mediate long-term transgene expression in cells *in vitro* and *in vivo* because of its low toxicity and immunogenicity [31]. These longer lasting vectors could have been used in

the study, thereby making it more functional and indicating its more widespread potential.

Discussion

In this study, we constructed a recombinant hLF adenovirus and examined the recombinant hLF expression in milk by infusing the viruses into rabbit mammary glands. Western blot analysis demonstrated that the human recombinant lactoferrin was expressed in the milk of rabbits. Further ELISA experiments suggested that it is possible to obtain high-level expression of recombinant human lactoferrin in the milk of rabbits through the direct instillation of the recombinant adenovirus to the mammary gland. In previous attempts, the recombinant human lactoferrin had been expressed in transgenic cows (level of 2 mg/ml, [29]), mammalian BHK cells [13], silkworm larvae (192 μg/ml level [16]), *Spodoptera frugiperda* cells, and tobacco cells [19, 25, 26]. In the present study, we demonstrated that a high expression level of hLF in rabbits (level of 2.3 mg/ml) was obtained. Although the transgenic animals also could provide a higher expressed rhLF in milk, this transgenic system still has some serious limitation in current technology, especially its difficult use for domestic species. The method established here is easy-handling and less time required (four months or less), and therefore could be more effective for production of rhLF or other biopharmaceutical interests. In addition, recent development of mammalian cell culture technology allows one to achieve the high expression level of recombinant proteins [3, 22]. However, there are no doubts that the possibility of expression of high levels of recombinant proteins in milk through the use of adenoviral vectors has noticeable advantages over traditional technologies. For example, the method is simple and can be directly applied to large livestock. Furthermore, the method is easy to handle and allows one to obtain several kinds of recombinant proteins simultaneously expressed in the milk.

In previous studies, the methods of direct target gene transfer to the mammary glandular epithelium for delivering secreted proteins to the milk of animals have been described. These include the use of retroviral vectors, by which Archer *et al.* [1] reported a maximum expression level of hGH (118 ng/ml) at the early lactation in goats, followed by a rapid decrease below 20 ng/ml until the end of the experiment. In another attempt, Fan and coworkers [6] used an adenoviral vector carrying a modified lysostaphin gene to directly infuse the dry mammary gland of goats; however, the protein expression level of the target gene was very low. Hens *et al.* [9] have reported that the mammary gland infused with a plasmid DNA complex obtained expression levels of hGH throughout lactation. However, the expression levels did not exceed 600 ng/ml. Recently, Sanchez *et al.* [27] obtained the high expression of hGH in milk at above 2 mg/ml in mice and up to 0.3 mg/ml in goats. In contrast to these earlier studies, our data strongly suggested that it is possible to obtain high-level expression of exogenous protein in the milk of rabbits. In this study, we were able to obtain high human lactoferrin expression levels during the 10 days of lactation in rabbit milk by simple infusing the adenoviral vector through the nipple canal. This is the first report of this way to obtain high expression of human lactoferrin at above 2 mg/ml in the milk of rabbits.

Although we obtained the high expression level of target protein in the milk of rabbits, the hLF was transiently secreted into the milk for 10 days. The short-term expression of the hLF might result from limited mammary epithelial cells to be infected. Another possible explanation was that the viral vector used in this study, which might express other viral proteins encoded in the remaining viral genome, might be subject to host-immune-mediated destruction of infected cells [34]. However, some new adenoviral vectors could be expected to overcome this problem, by which a long-term target gene expression would be possible in gene therapy assays [12].

In conclusion, we described a simple, fast, and high-expression method of production of recombinant human lactoferrin in milk. Although the rhLF is transient compared with traditional transgenic animals, it is probably one of the most economical and efficient ways for hLF production. We believe that our method will make it easier for other investigators to obtain large quantities of rhLF. This method may also be suitable to the study of the biological characteristics of recombinant proteins expressed in the milk of different species.

References

1. Archer, J. S., W. S. Kennan, M. N. Gould, and R. D. Bremel. 1994. Human growth hormone (hGH) secretion in milk of goats after direct transfer of the hGH gene into the mammary gland by using replication-defective retrovirus vectors. *Proc. Natl. Acad. Sci. USA* **91:** 6840–6844.
2. Arnold, R. R., M. F. Cole, and J. R. McGhee. 1977. A bactericidal effect for human lactoferrin. *Science* **197:** 263–265.
3. Bae, G. W., D. W. Jeong, H. J. Kim, G. M. Lee, H. Park, T. B. Choe, S. Kang, I. Y. Kim, and I. H. Kim. 2006. High productivity of t-PA in CHO cells using hypoxia response element. *J. Microbiol. Biotechnol.* **6:** 695–703.
4. Bellamy, W., M. Takase, H. Wakabayashi, K. Kawase, and M. Tomita. 1992. Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. *J. Appl. Bacteriol.* **73:** 472–479.
5. Crouch, S. P., K. J. Slater, and J. Fletcher. 1992. Regulation of cytokine release from mononuclear cells by the iron-binding protein lactoferrin. *Blood* **80:** 235–240.
6. Fan, W., K. Plaut, A. J. Bramley, J. W. Barlow, S. A. Mischler, and D. E. Kerr. 2004. Persistency of adenoviral-mediated

lysostaphin expression in goat mammary glands. *J. Dairy Sci.* **87:** 602–608.
7. Hashizume, S., K. Kuroda, and H. Murakami. 1983. Identification of lactoferrin as an essential growth factor for human lymphocytic cell lines in serum-free medium. *Biochim. Biophys. Acta* **763:** 377–382
8. He, T. C., S. Zhou, L. T. da Costa, J. Yu, K. W. Kinzler, and B. Vogelstein. 1998. A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA* **95:** 2509–2514.
9. Hens, J. R., M. D. Amstutz, F. L. Schanbacher, and I. H. Mather. 2000. Introduction of the human growth hormone gene into the guinea pig mammary gland by *in vivo* transfection promotes sustained expression of human growth hormone in the milk throughout lactation. *Biochim. Biophys. Acta* **1523:** 161–171.
10. Hock, K. S., J. M. Milne, P. A. Grieve, D. A. Dionysius, and R. Smith. 1997. Antibacterial activity in bovine lactoferrin-derived peptides. *Antimicrob. Agents Chemother.* **41:** 54–59.
11. Jeng, M. H., C. Kao, L. Sivaraman, S. Krnacik, L. W. Chung, D. Medina, O. M. Conneely, and B. W. O. Malley. 1998. Reconstitution of estrogen-dependent transcriptional activation of an adenoviral target gene in select regions of the rat mammary gland. *Endocrinology* **39:** 2916–2925.
12. Kreppel, F. and S. Kochanek. 2004. Long-term transgene expression in proliferating cells mediated by episomally maintained high-capacity adenovirus vectors. *J. Virol.* **78:** 9–22.
13. Legrand, D., V. Salmon, B. Coddeville, M. Benaissa, Y. Plancke, and G. Spik. 1995. Structural determination of two N-linked glycans isolated from recombinant human lactoferrin expressed in BHK cells. *FEBS Lett.* **365:** 57–60.
14. Levay, P. F. and M. Viljoen. 1995. Lactoferrin: A general review. *Haematologica* **80:** 252–267.
15. Liang, Q. and T. Richardson. 1993. Expression and characterization of human lactoferrin in yeast *Saccharomyces cerevisiae*. *J. Agric. Food Chem.* **41:** 1800–1807.
16. Liu, T., Y. Z. Zhang, and X. F. Wu. 2005. High level expression of functionally active human lactoferrin in silkworm larvae. *J. Biotechnol.* **118:** 246–256.
17. Lonnerdal, B. and S. Iyer. 1995. Lactoferrin: Molecular structure and biological function. *Annu. Rev. Nutr.* **15:** 93–110.
18. Mazurier, J., D. Legrand, W. L. Hu, J. Montreuil, and G. Spik. 1989. Expression of human lactotransferrin receptors in phytohemagglutinin-stimulated human peripheral blood lymphocytes. Isolation of the receptors by antiligand-affinity chromatography. *Eur. J. Biochem.* **179:** 481–487.
19. Mitra, A. and Z. Zhang. 1994. Expression of a human lactoferrin cDNA in tobacco cells produces antibacterial protein(s). *Plant Physiol.* **106:** 977–981.
20. Nibbering, P. H., E. Ravensbergen, M. M. Welling, L. A. van Berkel, P. H. van Berkel, E. K. Pauwels, and J. H. Nuijens. 2001. Human lactoferrin and peptides derived from its N terminus are highly effective against infections with antibiotic-resistant bacteria. *Infect. Immun.* **6914:** 69–76.
21. Nuijens, J. H., P. H. van Berkel, and F. L. Schanbacher. 1996. Structure and biological actions of lactoferrin. *Gland. Biol. Neoplasia* **1:** 285–295.
22. Park, J. H., S. R. Yu, J. S. Yoon, and K. H. Baek. 2005. High-level expression of recombinant human bone morphogenetic protein-4 in Chinese hamster ovary cells. *J. Microbiol. Biotechnol.* **5:** 1397–1401.
23. Patton, S., U. Welsch, and S. Singh. 1984. Intramammary infusion technique for genetic engineering of the mammary gland. *J. Dairy Sci.* **67:** 1323–1326.
24. Russell, T. D., A. Fischer, N. E. Beeman, E. F. Freed, M. C. Neville, and J. Schaack. 2003. Transduction of the mammary epithelium with adenovirus vectors *in vivo*. *J. Virol.* **77:** 5801–5809.
25. Salmon, V., D. Legrand, B. Georges, M. C. Slomianny, B. Coddeville, and G. Spik. 1997. Characterization of human lactoferrin produced in the baculovirus expression system. *Protein Expr. Purif.* **9:** 203–210.
26. Salmon, V., D. Legrand, M. C. Slomianny, I. el Yazidi, G. Spik, V. Gruber, P. Bournat, B. Olagnier, D. Mison, M. Theisen, and B. Merot. 1998. Production of human lactoferrin in transgenic tobacco plants. *Protein Expr. Purif.* **13:** 127–135.
27. Sanchez, O., J. R. Toledo, M. P. Rodriguez, and F. O. Castro. 2004. Adenoviral vector mediates high expression levels of human growth hormone in the milk of mice and goats. *J. Biotechnol.* **114:** 89–97.
28. Toledo, J. R., O. Sanchez, S. R. Montesino, G. Y. Fernandez, M. P. Rodriguez, and J. A. Cremata. 2005. Differential *in vitro* and *in vivo* glycosylation of human erythropoietin expressed in adenovirally transduced mouse mammary epithelial cells. *Biochim. Biophys. Acta* **1726:** 48–56.
29. van Berkel, P. H., M. M. Welling, M. Geerts, H. A. van Veen, B. Ravensbergen, M. Salaheddine, E. K. Pauwels, F. Pieper, J. H. Nuijens, and P. H. Nibbering. 2002. Large-scale production of recombinant human lactoferrin in the milk of transgenic cows. *Nat. Biotechnol.* **20:** 484–487.
30. van der Strate, B. W., L. Beljaars, G. Molema, M. C. Harmsen, and D. K. Meijer. 2001. Antiviral activities of lactoferrin. *Antiviral Res.* **52:** 225–239.
31. Vorland, L. H. 1999. Lactoferrin: A multifunctional glycoprotein. *APMIS* **107:** 971–981.
32. Wagner, K. U., R. J. Wall, L. St. Onge, P. Gruss, A. Wynshaw-Boris, L. Garrett, M. Li, P. A. Furth, and L. Hennighausen. 1997. Cre-mediated gene deletion in the mammary gland. *Nucleic Acids Res.* **25:** 4323–4330.
33. Ward, P. P., C. S. Piddington, G. A. Cunningham, X. Zhou, R. D. Wyatt, and O. M. Conneely. 1995. A system for production of commercial quantities of human lactoferrin: A broad-spectrum natural antibiotic. *Biotechnology (NY)* **13:** 498–503.
34. Yang, J., T. Tsukamoto, N. Popnikolov, R. C. Guzman, X. Chen, J. H. Yang, and S. Nandi. 1995. Adenoviral-mediated gene transfer into primary human and mouse mammary epithelial cells *in vitro* and *in vivo*. *Cancer Lett.* **98:** 9–17.
35. Zhang, G. H., D. M. Mann, and C. M. Tsai. 1999. Neutralization of endotoxin *in vitro* and *in vivo* by a human lactoferrin-derived peptide. *Infect. Immun.* **67:** 1353–1358.