Original Article

Efficient Human Growth Hormone Gene Expression in the Milk of Non-transgenic Goats

(adenovirus vector / human growth hormone / mammary gland / milk / goat)

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Abstract. Heterogenous expression of recombinant proteins in milk of livestock at a large scale is very labour-intensive to be achieved with current transgenic animals, and usually seen as time-consuming, expensive and technically most challenging. Here we describe a convenient system for transient production of recombinant human growth hormone and its extensive use in recombinant protein production for therapeutic purposes. In this study, an adenoviral vector containing the *GFP* gene and *hGH* gene was constructed for direct infusion into the epithelium of mammary glands of goats via the teat canal during the period of natural lactation. Western-blot analysis of milk samples obtained from all of the viral-treated founders indicated that the recombinant hGH (rhGH) was secreted into the milk of the goats. The concentrations of rhGH in milk ranged from 0.6 to 2.4 mg/ml and lasted for more than 10 days during lactation. These data suggest that it is possible to produce larger amounts of recombinant human growth hormone in the milk of livestock animals by using replication-defective adenoviruses.

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Abbreviations: CAR – coxsackie-adenovirus receptor, FBS – foetal bovine serum, GFP – green fluorescent protein, GME – goat mammary epithelial, hGH – human growth hormone, MEM – minimal essential medium, PBS – phosphate-buffered saline, PVDF – polyvinylidene fluoride, rhGH – recombinant hGH.

Introduction

High-level expression of recombinant protein in the animal mammary gland continues to be a desirable but still not easy goal (Houdebine, 2000; Bösze et al., 2008). The bioactivity of many proteins requires post-translational modifications such as glycosylation and carboxylation. These complex modifications may not occur in bacterial systems in such a way as to yield proteins of desired potency. As a result, proteins produced in bacteria may have to be chemically manipulated before recombinant proteins can be used for therapeutic or industrial purposes (Walsh and Jefferis, 2006). Using the transgenic animals' mammary gland technique, mammary epithelial cells have been engineered to secrete proteins of therapeutic interest such as human protein C and tissue plasminogen activator, important mediators of haemostasis and coagulation, and human a1-antitrypsin, which is involved in emphysema (Mikuš et al., 2001; Houdebine, 2005).

The expression of foreign proteins in milk of transgenic animals has most often been accomplished by cloning DNA encoding the protein of interest downstream of DNA fragments that contain defined transcriptional modules (Houdebine, 2000; 2005; Bösze et al., 2008). However, this strategy has not always produced large quantities of proteins because essential regulatory sequences are lacking in the transgenic DNA (Rosen et al., 1996). Furthermore, the high cost and uncertain results have made this method very hard to be applied in recombinant protein production.

Replication-defective adenoviral vectors have been used successfully to deliver target genes into the mammary gland in gene therapy (Fan et al., 2002; 2004; Peebles et al., 2004; Douglas, 2007). However, most of the target gene expression in the mammary epithelial cells is very low and inefficient. An alternate route of introducing target genes into the mammary gland has recently been demonstrated (Sánchez et al., 2004; Han et al., 2007). In the present study, the pAd-hGH recombinant adenoviral vector was used to transfer the target gene for human growth hormone (*hGH*) and green fluorescent

protein (*GFP*) reporter gene into goat mammary epithelial cells *in vitro* and *in vivo*. Concentrations of hGH in the milk from mammary gland of adenovirus-transduced goats were high, ranging from 0.6 to 2.4 mg/ml in all treated goats. The recombinant adenoviral vector containing the *hGH* gene used in this study was sufficient to target recombinant hGH (rhGH) expression to a high level into the mammary gland of lactating animals.

Material and Methods

Cells and Culture

Unless otherwise stated, all chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO). Human embryonic kidney (HEK) 293 cells were purchased from Microbix Biosystems Inc. (Toronto, Canada) and grown in minimal essential medium (MEM), supplemented with 10% (v/v) foetal bovine serum (FBS), 1% non-essential amino acids, 2 mM L-glutamate and 1% penicillin-streptomycin. Primary goat mammary epithelial (GME) cells were prepared as described previously (Han et al., 2007). The GME cells were maintained in MEM supplemented with 10% FBS, epidermal growth factor (10 ng/ml), insulin (10 μg/ml) and 1% penicillin-streptomycin. All cells were kept at 37 °C with 5% CO_2/95% air.

Construction of Viral Vectors

First, the *hGH* genomic DNA was cloned from an aborted foetus genome by using the PCR method. The primers used for PCR amplification were designed as follows, forward: TGGTACCATGGCTACAGGTAAG, reverse: 5′-CGCTCGAGCTAGAAGCCACAG-3′. To facilitate downstream subcloning of PCR products, we added *Kpn*I and *Xho*I (underlined) sites into 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 denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min 30 s, and ended at 72 °C for 7 min. The PCR products about 1.5 kb *Kpn*I/*Xho*I hGH fragment were cloned into the corresponding sites of the plasmid pcDNA3.1 (+) (Invitrogen, Carlsbad, CA) and sequenced. The plasmid was designated as p3gh. Second, we designed two primers (forward: 5′-TTAAGCTTACCATGGTGAGCAAGGGCGAG-3′, reverse: 5′-CCGATATCTTACTTGTACAGCTCGTCCATG-3′) to amplify the 735 bp *Hind*III/*Eco*RV (underlined) fragment containing the *GFP* gene from pEGFP-N1 vector (TaKaRa, Kyoto, Japan). Then the fragment containing the complete sequences for GFP was cloned into the plasmid pIRES (TaKaRa). The resulting plasmid was pIRg. Finally, the *Nhe*I and *Xho*I fragment containing the *hGH* DNA from the plasmid p3gh was cloned into the corresponding sites of the pIRg vector. The resulting plasmid was pIRgh.

The replication-defective adenovirus vector pAd-hGH was obtained by using the AdEasy adenoviral vector system. The procedures of AdEasy system were described in detail earlier (He et al., 1998). The 2.9-kb *Pme*I/*Not*I fragment from the plasmid pIRgh containing *hGH* and *GFP* genes was cloned into the pShuttle-CMV vector (Stratagene, La Jolla, CA). The resulting plasmid was transformed to the AdEasy bacteria containing the adenovirus type 5 genome deleted for E1 and E3 regions, yielding the pAd-hGH recombinant adenovirus vector. Fig. 1 shows the plasmid constructs used in this study.

Virus Production and Assay

The pAd-hGH vector was linearized with *Pac*I and purified by the commercial purification kits (Qiagen, Venlo, The Netherlands) 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 sterile phosphate-buffered saline (PBS). The cells were lysed by four cycles of freezing/thawing. Cell lysate was centrifuged at 2500 *g* for 5 min at 4 °C. The final viral stock was purified by the commercial kit (Qiagen) according to the manufacturer's instructions. Since there is a reporter marker gene in the pAd-hGH vector,

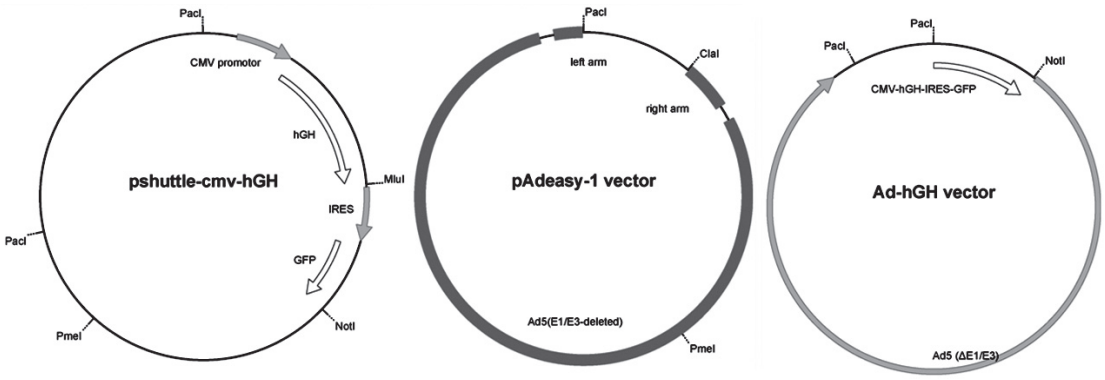

Fig. 1. Construction of recombinant adenoviral vectors containing the human growth hormone gene.

the titre of the recombinant adenovirus preparation was determined by GFP expression on semi-confluent HEK 293 cells. The final virus stock was stored at −70 °C until use.

Experimental Animals

Adult female goats (13 ± 0.5 kg) were bred from a local goat farm and kept in the animal facility at the school of biological engineering, the University of Yanshan. All animal procedures were approved by the Animal Care and Use Committee of the University of Yanshan.

Infusion of Recombinant Adenoviruses

For all mammary infusions and sample collections, teats were routinely wiped with 70% alcohol and iodine to prevent mammary gland infection. By using a syringe, the recombinant adenoviruses were infused directly into the left mammary gland of each goat. The left ventral mammary gland of each goat was infused with a different volume of viral stocks, ranging from 300 ml to 600 ml solution (containing 1×10^9 PFU/ml virus), and determined by the internal capacity of the gland. As an intra-animal control, the right mammary glands received the same volume of sterile PBS.

The treated goats 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 until use.

Western Blot Analysis of the hGH

Protein samples were mixed with equal volumes of loading buffer (10% β-mercaptoethanol, 0.2% SDS), and boiled for 5 min. After separation in 12% SDS-PAGE, the proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) using a semi-dry transfer method. The membrane was blocked with 5% non-fat 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 non-fat dried milk and mouse anti-human growth hormone monoclonal antibody for 1 h at 37 °C. The membrane was then washed three times (each for 10 min) with PBST followed by 1 h incubation of horseradish peroxidase-labelled goat anti-mouse IgG antibody at room temperature. After four times of washing with PBST, the hGH was detected by using the ECL kit (Amersham, Chalfont St. Giles, UK).

Determination of the hGH by ELISA

Concentration of the rhGH in milk serum samples was measured by an enzyme-linked immunosorbent assay (ELISA), using a commercially available test kit (TPI Inc., Washington, D. C.). Sera (50 µl) and the positive control were added to the precoated wells, then 50 µl enzyme-linked conjugates were instilled into the wells, which were incubated for 30 min at 37 °C. Freshly prepared O-phenylenediamine (OPD) solution was added to the wells and incubated for 15 min at room temperature. After blocking the reaction, the absorbance was measured at 450 nm by a Thermo Labsystems Multiskan Mk3 plate reader (Thermo, Vantaa, Finland). The concentration of hGH was calculated from the OD_{450} values based on the hGH ELISA standard curve.

Results and Discussion

Viral Vector Production and hGH Expression in Vitro

As a first step in viral vector construction, we used the *GFP* gene as a reporter gene to facilitate recombinant adenovirus production. The pAd-hGH vector contains an internal ribosome entry site (IRES) sequence which allows expression of two different genes of interest at high levels (Rees et al., 1996). Under the fluorescence microscope, the GFP protein was found in 293 cells infected with the pAd-hGH vector (data not shown). Since the *GFP* gene was cloned downstream of the IRES sequence controlled by the same CMV promoter, the expressed GFP indicated the expression of rhGH protein in this study. The rhGH levels in cellular supernatant were determined after 48-h incubation. By using the ELISA method, the result showed that the rhGH was expressed at a high level, up to 0.422 mg/ml in cell medium from infected 293 cells. However, there was no rhGH expression in the uninfected 293 cells (data not shown). These data suggested that the viral vector used in this study could be further applied to infect goat mammary epithelial cells.

Recombinant adenoviruses have been shown to produce a high transduction efficacy in goat mammary epithelial cells in separate studies (Sánchez et al., 2004; Han et al., 2007). To confirm the ability of the viral vector to transduce mammary cells, the GME cells were infected at a MOI of 25. The rhGH level in cellular supernatants was determined after 48-h infection. Twenty-four hours after the infection, the GFP fluorescence was also found in the infected GME cells (data not shown). This result suggests that the pAd-hGH vector could be used *in vivo* in the goat mammary gland epithelial cells and it is possible to obtain high transduction.

Infusion of Viral Vector into Goat Mammary Glands

It has been observed that the recombinant adenoviruses could induce high-level expression of heterogeneous protein in the milk of goats (Toledo et al., 2006). We wanted to determine whether the pAd-hGH vector could achieve efficient delivery of the *hGH* gene into the mammary gland epithelial cells *in vivo*. The viral stock containing 1×10^9 PFU/ml was then infused directly into the left mammary glands of three goats. As a control, the right glands of the goats received the same volume of

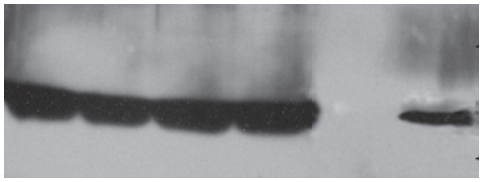

Fig. 2. Western blot analysis of rhGH expression in milk from viral vector-infected mammary gland. Proteins were run on a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a PVDF membrane. Protein samples were checked for their compatibility with Western blot analysis using an anti-human growth hormone antibody. Lanes 1–4, milk serum samples from infected mammary glands of goats during lactation days 2 to 5; lane 5, milk serum samples from uninfected mammary glands of goats during lactation days; lane 6, commercially available rhGH protein standards.

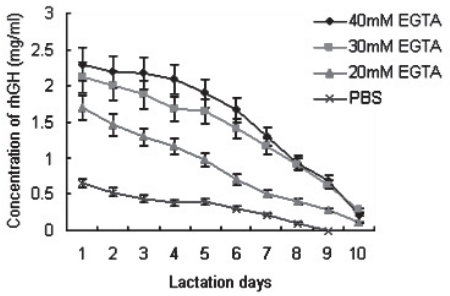

Fig. 3. Effect of EGTA on the adenoviral transduction efficiency of goat mammary epithelium. Four groups of goats were used and treated with different doses of EGTA: 20, 30 and 40 mM. The PBS treatment served as a control. Milk sera were determined by using the ELISA method. Bars represent the mean and SD.

sterile PBS. The concentration of rhGH expression was determined by using the immunoassay method on indicated days. Forty-eight hours after the infusion, the concentration of rhGH for goats was determined to be 0.241 mg/ml, 0.311 mg/ml, and 0.196 mg/ml, respectively. However, there was no detectable rhGH in the PBS-treated mammary glands. The recombinant hGH was detected in the milk by Western blot in all virus-treated goats (Fig. 2). Western blot analysis indicated that the hGH recombinant protein expressed in goat milk showed a very similar migration with the commercial hGH from Sigma. In a separate study, Sánchez et al. reported that in goats the viral infection occurs less efficiently than in mice. This lower infection could be caused by the lower affinity of adenoviral vector for goat Coxsackie-adenovirus receptor (CAR). On the other hand, the lower expression in milk of goats could be a result of the lower permeability of the mammary secretory epithelium during the viral vector infusion. This result suggested that the expressed proteins in the treated mammary gland apparently did not reach their maximal levels in lactation, and could still have a potential space for recombinant protein production. Indeed, further experiments are needed to investigate the higher-level expression of recombinant proteins in the milk of goats.

Production of rhGH in the Milk of Goats

In order to enhance the transfection efficiency of mammary gland, the ruminant mammal can be treated to loosen the apical tight junctions of the epithelial cells of the cap facilitating the flow of the adenoviral vector across the epithelium (Chu et al., 2001; Johnson et al., 2003). For this reason, the calcium chelator ethylene glycol-bis (2-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA) was used in this study through direct infusion into the mammary gland with adenoviral vector infusion. Nine goats were divided into three groups in this experiment, and each group had three goats at random. The mammary glands of goats were filled with a solution of viral vector supplemented with 20 mM, 30 mM and 40 mM of EGTA, respectively. As seen in Fig. 3, the glands infused with the viral vector containing EGTA showed a significantly higher expression of hGH compared to that of PBS-viral solution ($P < 0.05$). The highest level of hGH in EGTA-treated group was 2.3 mg/ml on day 1 of milking and after that, the expression levels of hGH dropped drastically between day 4 and 7 of lactation. The results also showed that 40 mM EGTA was an optimal dose for mammary glands of goats, but there is no significant difference compared to the 30 mM-dose treatments ($P > 0.05$). Our results are also in agreement with previous reports showing that EGTA could enhance gene transfer by modulating paracellular permeability and result in high-efficiency adenovirus transfection *in vivo* (Chu et al., 2001; Sánchez et al., 2004).

Analysis of Goat Milk

In all the above experiments, no symptoms of mastitis were found in the mammary gland of goats. Milk appeared normal throughout the lactation. For all goats, the volume of milk obtained from each udder half was about 100 ml on the first day of lactation and became normal gradually. As shown in Fig. 4a, production of milk obtained from each udder was variable and appeared increasing daily (Fig. 4a). Interestingly, the concentration of rhGH expression patterns for each goat seemed to be different by using ELISA detection (Fig. 4b). This variance of concentration is probably the result of individual differences existing in animals. In addition, no rhGH expression was observed in the milk of goats in each right mammary gland.

In our study, we successfully constructed the pAd-hGH vector, and this is the first generation of adenoviral vectors that can infect dividing and non-dividing cells, and where the viral genome stays episomal and is not integrated into the host genome. This feature and their ability to infect a broad range of cells make such adenoviruses promising and versatile vectors for gene therapy (Peebles et al., 2004; Douglas, 2007). Furthermore, they can be produced to very high titres and therefore attain

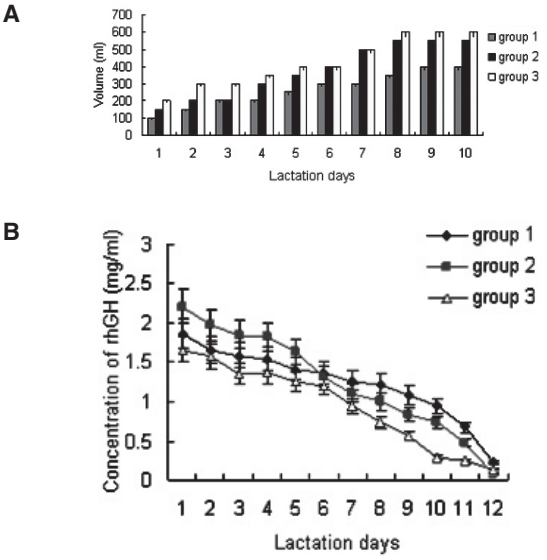

Fig. 4. (a): Volumes of milk per udder half/day. (b) The variation of rhGH expression patterns in transduced mammary gland of goats. Bars represent the mean and SD.

high levels of transgene expression. Our results also suggest that the viral vector could result in high-level rhGH expression in the milk of goats. Although the expression of recombinant protein in milk is transient, the method described here probably represents one of the most economical ways to obtain heterogeneous proteins in the milk of livestock. Furthermore, the methods described here display high efficiency compared to traditional transgenic animals. The most serious limitation in the transgenic animal technology for livestock species is the time required and the expense of producing founder animals either by pronuclear microinjection or by nuclear cloning (Lipiński et al., 2003; Salamone et al., 2006).

In previous studies, the direct *in vivo* transduction of the mammary gland has been proved to be a less expensive and simpler alternative to produce recombinant proteins in the mammary epithelial cells (Fan et al., 2002; 2004; Sánchez et al., 2004; Toledo et al., 2006; Han et al., 2007). For hGH production, the expression levels in previous studies were very low. By use of polyion complexes through the teat or nipple canal into the secretory tissue, Hens et al. (2000) obtained a maximum expression of 550 ng/ml in the milk of guinea pigs. By use of replication-defective retroviruses, Archer et al. (1994) obtained the highest expression of 118.6 ng/ml at the start of lactation. In addition, other expression systems also were able to achieve hGH expression, but both obtained low-expressed target proteins (0.2–0.6 μg/ml and 0.24 μg/ml) (Kajino et al., 1997; Hawkins and Nakamura, 1999). Compared to previous experiments, we were able to obtain a higher level of expressed hGH in the milk of goats (more than 2 mg/ml). In addition, the method described here is probably an easier and faster way to produce recombinant proteins in the milk of livestock animals.

In conclusion, we describe here a high-yield transient expression system for the production of hGH in transfected mammary glands of goats. Our results indicate that the use of viral vectors for high-yield production of human therapeutic proteins or functional gene expression analysis in milk of goats by transient expression provides an attractive alternative to production protocols using adenoviral vectors in ruminants.

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Zengsheng Han and Shuyun Wu contributed equally to this work.

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