REPORT

New procedure for the production of biopharmaceutical proteins in the milk of non-transgenic animals

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ABSTRACT

The production of large quantities of complex proteins with biopharmaceutical purposes is the main drawback for their more extensive use. Here we demonstrated that through the direct transduction of the mammary glandular epithelium by means of adenoviral vectors it is possible to promote high expression levels of recombinant proteins in the milk of non-transgenic animals. Through this approach we were able to express high levels of human growth hormone and human erythropoietin in the milk of both mice and goats. We found that the expression levels were closely dependent on both the degree of differentiation of the secretory epithelium and the adenoviral dose used. Direct transduction of the mammary gland seems to be a suitable alternative to express high levels of complex proteins in milk and it may eventually constitute a platform for the development of new biotechnological processes.

Keyword: adenovirus, recombinant protein, milk, mammary gland

Introduction

The mammary gland has been identified as a suitable alternative to produce biopharmaceutical and nutritional proteins [1, 2], that are synthesized in the mammary epithelial cells and secreted into the milk from which the purification process is relatively simple. Transgenesis has been extensively used for the genetic modification of farm animals to produce human recombinant proteins in milk [3]. However, drawbacks in this technology include the technically difficult procedure required, its inefficiency when applied to large livestock, the length of time between the incorporation of foreign DNA and harvesting the exogenous protein, and the health risk for animals due to the ectopic expression of transgenes.

The direct *in vivo* transfection of the mammary gland has been proposed as a faster and more inexpensive alternative to target the expression of a heterologous gene to the secretory mammary epithelial cells [4, 5]. In this way, the mammary epithelium has been transduced by polyion complexes [6], receptor-mediated endocytosis [7], gene-gun devices [8, 9], retroviral vectors [10-12] and adenoviral vectors [13-17]. Only in a few of these studies the recombinant protein was expected to be secreted in milk [6, 7, 11, 16], however the expression levels in all of them was very low, reaching in the best case hundreds of nanograms per milliliter [6, 16]. Although these expression levels may be enough for certain basic studies, they must be improved when large amounts of recombinant proteins are required.

Here we show that through the direct infection of the secretory mammary epithelium with a replication-defective adenoviral vector, it is possible to obtain high expression levels of foreign proteins in milk. The direct intramammary infusion of an adenoviral vector, harboring the human growth hormone gene (hGH), promoted the expression of the recombinant hormone at levels above 2 mg/mL in the milk of mice and up to 0.3 mg/mL in goat milk. Through the use of an adenoviral vector carrying the human erythropoietin (hEPO) cDNA it was possible to produce the recombinant protein at levels higher than 2 mg/mL in mouse milk and up to 2 mg/mL in goat milk.

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Materials and methods

Recombinant adenovirus

The replication defective adenoviral vectors were generated using the AdEasy system [18]. For the construction of the adenoviral vector containing the hGH expression cassette, the 2.2 kb BamH I/EcoR I hGH genomic fragment was cloned on the Bgl II and EcoR V sites of the pAdtrack-CMV vector. For the vector containing the hEPO expression cassette, a 0.6 kb Xho I/Hind III fragment containing the hEPO cDNA was cloned on the Sal I and Hind III sites of the pAdtrack-CMV vector. The resulting plasmids were recombined in BJ5183 electrocompetent bacteria, with the pAdEasy-1 vector containing the adenovirus type 5 genome deleted for E1 and E3 regions, yielding the AdhGH and the AdhEPO recombinant adenoviral genomes. Primary viral stocks were prepared by transiently transfecting the recombined AdEasy plasmids into host HEK-293 cells. The resulting vectors was named AdhGH and AdhEPO. Both vectors contain the gene of interest (hGH or hEPO) and a green fluorescent protein (GFP) coding sequence under the separate control of two cytomegalovirus (CMV) promoters. Adenovirus stocks were further amplified in HEK-293 cells, purified by a double cesium chloride gradient, titred by GFP expression on semi-confluent HEK-293 cells and expressed in gene transfer units (GTU). The concentrated stock was stored at -70 °C for later use.

In vitro expression assay

Mammary epithelial HC-11 cells were seeded in 12-well plates at a density of about 2.5 x 10^5 cells/cm^2 in DMEM supplemented with 10% fetal calf serum, epidermal growth factor (10 ng/mL) and insulin (10 µg/mL). After three days of confluence, the medium was replaced with fresh DMEM supplemented with insulin (5 µg/mL), and the cells were infected with the adenoviral vector at the desired multiplicity of infection (MOI). Seventy-two hours later the medium was collected and

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the supernatant tested for hGH content by SDS-PAGE, Western blot and ELISA.

Adenovirus infusion

Female mice were bred and the presence of a vaginal pug marked day 0 of gestation. Mice were anesthetized for adenovirus infusion by intraperitoneal injection of 80 µL of 25 mg/mL of pentobarbital sodium in saline. The four ventral mammary glands were cleaned with 70% ethanol and infused with 100 µL of a solution of PBS containing the desired amount of the adenoviral vector. For the infusion we used a glass micropipette, with a tip of about 50 µm, connected to a 1 mL syringe. At least three mice were used in each experimental group. They were milked on the indicated days, and milk letdown was induced by the injection of 0.3 U oxytocin. Milk samples from the same experimental group were pooled for analysis. The milk collected was diluted five times in the separation buffer (10 mM Tris-HCl pH8, 10 mM $CaCl_2$), after centrifugation at 12 000 rpm for 15 minutes, the milk serum was extracted and stored at -70 °C for later use.

Saanen goats were subjected to a two-week hormonal regime to induce mammogenesis and lactation [19]. Prior to adenoviral infusion, each half of the udder was flushed with PBS at 37 °C through the teat canal until the udder was full, the solution was removed by milking and the flushing step was repeated one or two more times until the solution was clear. The volume of the adenoviral vector infused was variable according to udder capacity. The day of adenoviral infusion as well as the adenoviral dose used is specified in the paper. Twenty-four hours after adenovirus instillation, udders were extensively milked to remove the infused solution. Milk collection began 48 hours after adenovirus inoculation and it was collected daily for eight to nine days.

Protein detection

Protein samples were separated on SDS-PAGE (13.5% acrylamide), then transferred onto nitrocellulose membranes on a semi-dry transfer. The membranes were subjected to Western blot analysis. The levels of hGH were determined using an hGH ELISA kit (Boehringer Mannheim, Germany), according to the manufacturer's protocol. The levels of hEPO were determined using hEPO ELISA (manufactured at the CIGB, Cuba), according to the manufacturer's protocol.

Results and discussion

In vitro expression assay in mammary epithelial cells

As a first step we constructed the adenoviral vectors AdhGH and AdhEPO. Both vectors contain simultaneously the gene of interest (hGH or hEPO) and a GFP coding sequence under the separate control of two cytomegalovirus (CMV) promoters. The incorporation of a GFP protein in this vector was designed to serve as a live marker for tracking infected cells or tissues in animal studies. It should be noted, however, that, with this dual cassette vector configuration, interference between the two expression units may lead to decreased levels of hGH expression relative to single-cassette vectors.

To assess the ability of this vector to transduce mammary epithelial cells, the murine HC-11 cell line was infected with the AdhGH vector at a MOI of 5, 25 and 125. Twenty-four hours after infection, GFP expression was detected in almost 100% of the cells in all the MOI assayed (data not shown); however the intensity of the fluorescence was variable and directly correlated with the viral load. Transduction of HC-11 resulted in high expression levels of hGH in the culture medium, both Coomassie blue-stained SDS/polyacrylamide gels and western blot revealed its presence as two protein bands, one major band of 22 kDa and a minor band migrating at 20 kDa (Figures 1A, B).

Although the expression levels of hGH in the culture medium was found to depend on the amount of the adenoviral vector used, a non-linear relationship was found between the adenoviral loads and the amount of hGH secreted in the medium (Figure 1C). The hGH levels dropped when the viral load was increased to 125 viral particles per cell. This slight fall in the expression level, seems to be the result of the high cell death observed during the medium harvest (data not shown). Cellular death, apparently associated to the cytopathic effect caused by the high viral dose, or by GFP accumulation, suggests the existence of an optimal viral dose over which cell damage precludes the expression of the foreign protein.

Murine HC-11 cells that are otherwise difficult to transfect by standard and commercial transfection

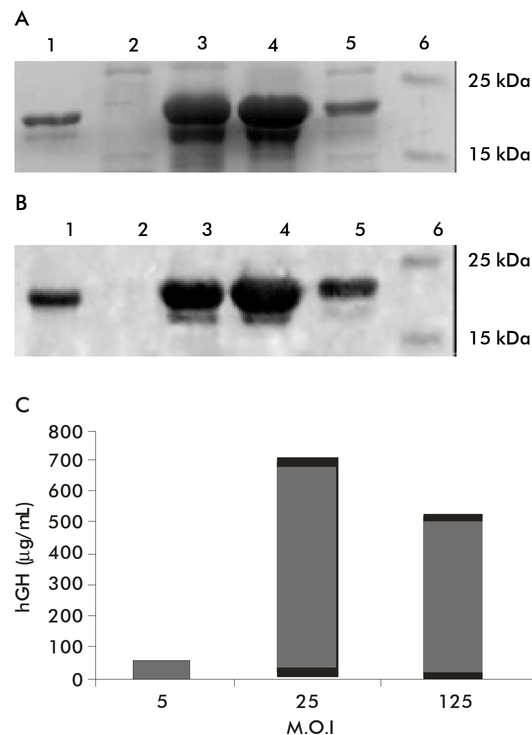

Figure 1. hGH expression after the infection of murine HC-11 cells with AdhGH. (A) Coomassie blue-stained SDS/polyacrylamide gel. (B) Western blot. (C) hGH expression levels as determined by ELISA. In both A and B Trichloroacetic acid-precipitated proteins from 150 µl of cell culture medium were loaded in each well. Lanes: 1)- Bacterially produced hGH, 2)-medium from non infected cells, 3)-MOI of 125, 4)-MOI of 25, 5)-MOI of 5, 6)-molecular weight marker.

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methods, became easily transduced with the adenoviral vector used in our experiments. This fact can be related to the wide expression of the coxsackievirus and adenovirus receptor (CAR) in these cells, which suggested that the same phenomenon is likely to occur *in vivo* in the mouse mammary gland.

Transduction of mouse mammary gland *in vivo*

Determination of the optimal time for adenovirus infusion

To determine the optimal period for adenovirus administration, we created four experimental groups according to the day of adenovirus instillation. The ventral mammary glands of mice were infused with 1x10^7 GTU per MG on days 13, 16, or 18 of gestation or on day 1 postpartum. Milk was collected on days 2, 5, 8 and 12 after parturition. Pectoral mammary glands were milked at the indicated days as a negative control.

To have a preliminary idea of the infection efficiency, a mouse was slaughtered on day 2 post-partum and small pieces of the mammary glandular tissue were observed under a fluorescence microscope. The presence of a high number of cells intensely fluorescent and arranged in form of bunch suggested that the infection occurred efficiently and that the secretory cells in the alveoli constitute the main target for viral transduction (Figure 2A).

The hGH was expressed in all four experimental groups (Figure 2B). Higher expression levels were obtained in the groups infused with the adenoviral vector during late pregnancy. In these animals, hGH was expressed at over 100 µg/mL, reaching 301 µg/mL on the second day of lactation in mice instilled on day 16 of pregnancy. Animals infused with the adenoviral vector at day 13 of pregnancy and day 1 post-partum showed the lowest expression levels. In these groups hGH was expressed within an order of magnitude lower than that obtained in the groups instilled during late pregnancy. None of the mice used in this experiment showed symptoms of mastitis. No hGH was detected in the milk from uninfected mammary glands.

In all four experimental groups the concentration of hGH gradually declined with time. This drop in the expression levels is in accordance with previous reports showing that the use of the ΔE1ΔE3 adenoviral vector only allow for a short-term expression, basically due to the immune response against the infected cells [20].

Correlation of the adenoviral vector dose with hGH expression levels

To investigate whether an increase of the viral dose could lead to an increase in the expression levels of hGH, the ventral mammary glands of mice on day 17 of pregnancy were infused with 1x10^7, 1x10^8 or 1x10^9 GTU/MG. Following parturition, milk was collected from day 2 to day 10 of lactation and tested for the presence of hGH. Pectoral mammary glands were milked as the intra-animal negative control.

The human growth hormone was detected in milk by both the Coomassie blue-stained SDS/polyacrylamide gels and Western blot in all three doses tested (Figure 3A-F). As judged by Western blot, hGH expressed in milk migrated as a major band of 22 kDa and two minor bands of about 19 and 12 kDa. The presence

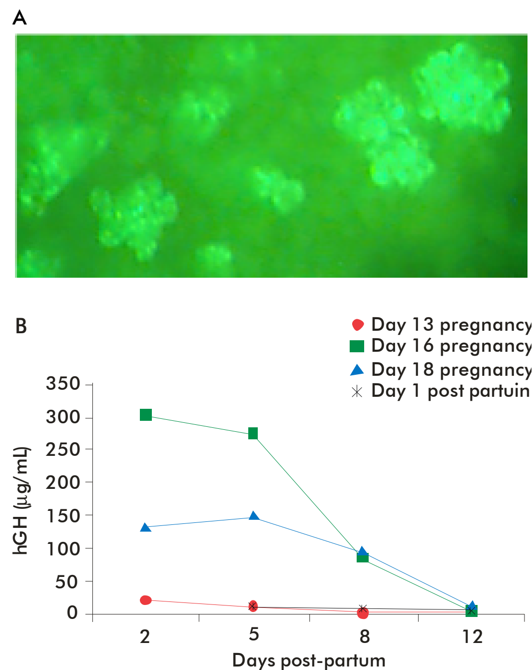

Figure 2. Adenoviral transduction of the mammary glandular epithelium. (A) GFP expression in the mammary gland of mice infused with AdhGH. The fluorescent cells are arranged in an aggregated form, which resemble the alveoli. (B) Time-course expression of hGH in mouse milk depending on the moment of adenoviral instillation.

of these immunoreactive bands is in agreement with previous studies showing that mRNA of hGH is differentially spliced when it is expressed in the mammary gland of transgenic mice [21].

The concentration of hGH in milk was clearly dependent on the adenoviral dosage (Figure 3G). Mice that received 10^8 GTU/MG expressed hGH in their milk at an average of 1.316 mg/mL, whereas the groups infused with 10^9 GTU/MG and 10^7 GTU/MG averaged 0.464 and 0.221 mg/mL respectively. In mice instilled with 10^8 GTU/MG, an hGH expression peak of 2.852 mg/mL was obtained at day 4 of lactation, this was about 8 times higher than that obtained on the same day in mice instilled with 10^7 GTU/MG. Infusion of 10^9 GTU/MG did not improve the expression levels of hGH. The highest expression detected in this group was of 1.642 mg/mL on day 2 of lactation and after that, the concentration of hGH dropped drastically between days 3 and 5 of lactation. This abrupt fall seemed to be a result of a massive death of the infected epithelium due to the cytopathic effect associated to the viral infection or to the strong cytotoxic response of the immune system against the infected cells.

Expression of hEPO in mouse milk

To assess the ability of the adenoviral vector to promote the expression of high complex glycoproteins in the milk of non-transgenic mice, 10^8 GTU of the AdhEPO vector were infused in the four ventral mammary glands of mice on day 17 of pregnancy. Following parturition, milk was collected from day 2 to day 8 of lactation and tested for the presence of hEPO. Pectoral mammary glands were milked as intra-animal negative control.

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The hEPO was expressed at high levels in all the mice infused with the adenoviral vector AdhEPO (Figure 4A, B). The protein followed an expression pattern similar to that shown by hGH. An expression peak was observed on day six post-partum when the hEPO level reached 3.5 mg/mL. However, the concentration of hEPO gradually declined with time, and on day 8 of lactation it was as low as 0.1 mg/mL. The Western blot assay revealed that mhEPO migrates as a compact band with a molecular weight of about 32-33 kDa, smaller than the acid fraction of CHO derived hEPO (CHO-hEPO), which migrated as a broad band ranging from 37 to about 42 kDa (Figure 4C).

The high electrophoretic mobility showed by mhEPO was in accordance with previous reports when the bovine β-lactoglobulin/hEPO fusion protein was produced in the milk of transgenic mice and rabbits [22]. The total N-deglycosylation assay showed that the difference in molecular weight was due to lower carbohydrate content in the molecule. Partial deglycosylation demonstrated that all three potential sites for N-glycosylation were occupied, which suggests that the underglycosylation observed in the mhEPO was due to a small size of the N-linked glycan chains (data not shown).

Transduction of the goat mammary gland *in vivo*

In order to determine whether the results obtained in mice could be extended to species of productive interest, we infused the AdhGH vector in the mammary gland of goats. One lactating and two hormonally induced to lactate goats were used. In the goats with the hormonally induced lactation, viral infusion was made in the late stage of the hormonal regime, a moment at which milk production was low, not exceeding 20 mL per mammary gland. After the treatment with corticosteroids [19] milk production increased up to 6-fold and it kept increasing gradually during the course of the experiment.

The expression of hGH in goat milk followed an expression kinetics very similar to that previously observed in mice (Figure 5B). The expression of hGH was transient and showed a significant dependence on both the viral dose and the degree of differentiation of the mammary epithelium. In goats with hormonally induced lactation, infusion of 10^9 GTU/mL allowed the expression of hGH at around 0.3 mg/mL at the start of milking, which was an order of magnitude higher than that obtained in the milk from mammary glands infused with 10^8 GTU/mL for the same time period. Between days 2 to 7 of milking, goats with hormonally induced lactation produced an average of 430 mL of milk/animal/day, and in mammary glands infused with 10^9 GTU/mL the total hGH expressed in this period was 530 mg. The infusion of a viral solution at 10^9 GTU/mL into the mammary gland of a goat in the third month of lactation allowed for the expression of about tenfold less hGH than that obtained in hormonally induced goats that were treated with a similar viral dose.

The lower expression level observed in the actively lactating goats seems to be the result of either the lower affinity of the adenoviral vector for the goat CAR, or of the lower permeability of the secretory epithelium during viral infusion. It is not clear whether the CAR are present on the apical surface of polarized mammary epithelial cells, however, previous studies have demonstrated that in polarized airway cells, CAR resides primarily on the basolateral membrane [23] and as a component of tight junctions [24]. During late pregnancy, progesterone withdrawal triggers the tight junction closure [25], with this impermeabilization, a deficiency of viral receptors on the apical cell surface may be considered as a barrier to the adenovirus infection from the apical side.

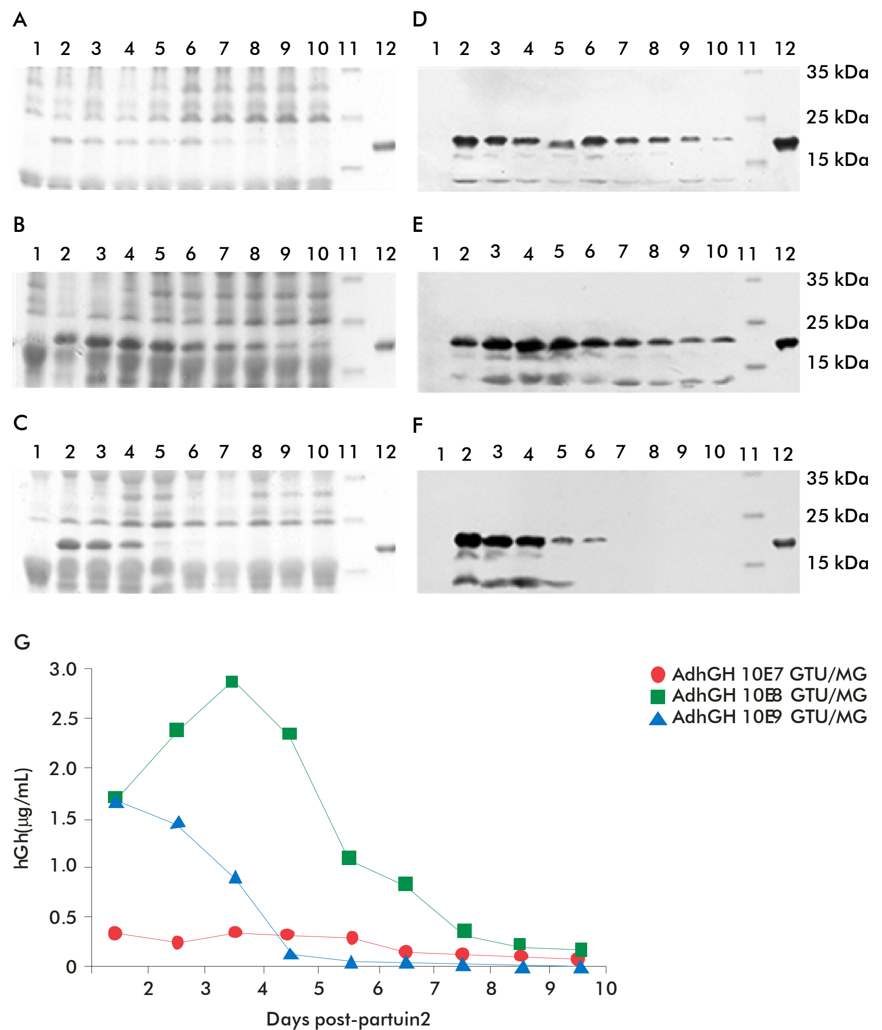

Figure 3. Effect of the adenoviral dosage on the expression levels of hGH. (A-C) Coomassie blue staining SDS gel electrophoresis. (D-F) Western blot of the same samples. Mice were infused with 107 GTU/MG (A, D); 108 GTU/MG (B, E) or 109 GTU/MG (C-F). Five microliters of milk serum were loaded in each well. Lanes: 1) Milk serum from uninfected mammary glands, 2-10)-milk serum samples from infected mammary glands of mice from day 2 to 10 post-partum, 11)-molecular weight marker, 12)-bacterially produced hGH. (G) Time-course of hGH expression on mice infused with different doses of AdhGH.

Temporal disruption of tight junctions

Since the low permeability of the secretory epithelium seems to be a determining factor for the inefficient infection of lactating goats; a temporal disruption of tight junctions through an EGTA treatment [26], may be a potential solution to this problem. With the aim of proving this hypothesis, four heifer goats were hormonally induced to lactate. Once concluded the

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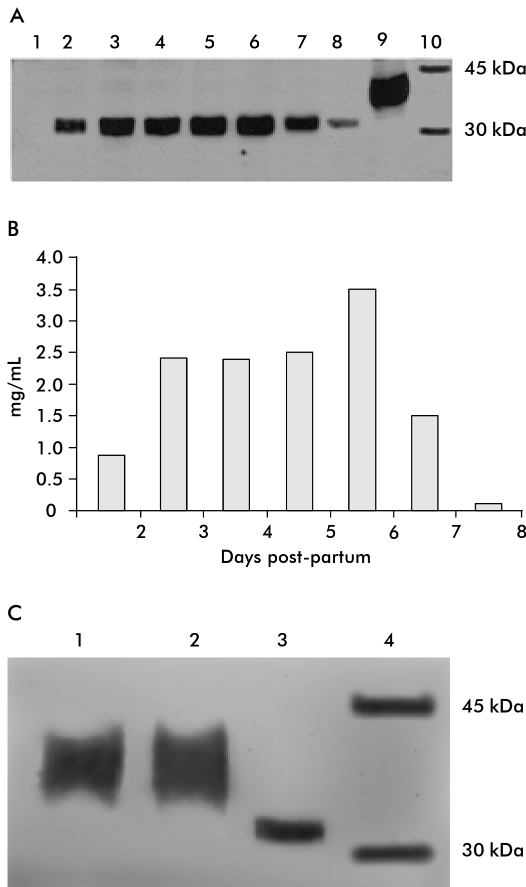

Figure 4. Effect of the adenoviral dosage on the expression levels of hGH. (A-C) Coomassie blue staining SDS gel electrophoresis. (D-F) Western blot of the same samples. Mice were infused with 107 GTU/MG (A, D); 108 GTU/MG (B, E) or 109 GTU/MG (C-F). Five microliters of milk serum were loaded in each well. Lanes: 1) Milk serum from uninfected mammary glands, 2-10)-milk serum samples from infected mammary glands of mice from day 2 to 10 post-partum, 11)-molecular weight marker, 12)-bacterially produced hGH. (G) Time-course of hGH expression in mice infused with different doses of AdhGH.

hormonal induction, the animals were milked for 30 days and the udders were massaged several times a day. During this period there was a noticeable increase both in udder size and in milk yields. The right half of the mammary glands were filled with PBS solution containing 10^9 GTU/mL of the AdEPO vector whereas the left half were infused with the same viral dose in a solution of PBS supplemented with 36 mM of EGTA.

In both experimental groups the hEPO expression followed an expression kinetic similar to that shown by hGH. As in mice, hEPO ran as a compact band slightly lower than the CHO-hEPO. Further analyses showed that the differences in size, as ocurring in mice, were due underglycosylation in the milk derived molecule (Figure 6A). The half of the mammary gland infused with the viral inoculums containing EGTA showed an expression of hEPO about two fold higher than that of its counterpart infused with a solution devoid of EGTA (Figure 6B). Although there was a noticeable difference on the hEPO expression levels between both groups during the first days of milking,

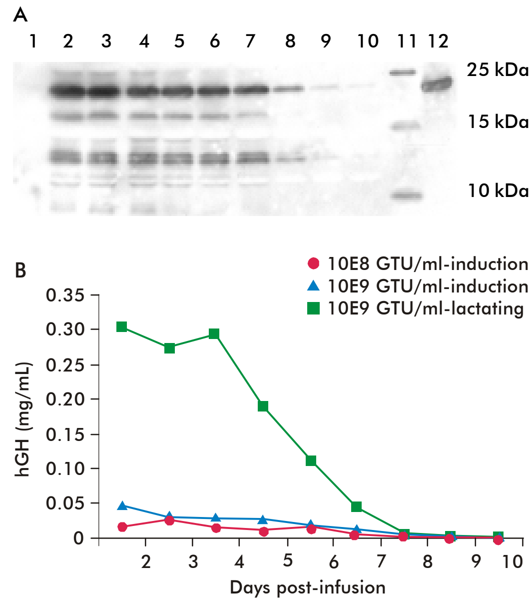

Figure 5. Expression of hGH in the milk of goats. (A) Western blot of milk serum from a goat mammary gland infused with a solution containing 109 GTU/mL of the adenoviral vector AdhGH. Five microliters of milk serum were loaded in each well. Lanes: 1) Milk serum from uninfected mammary glands, 2-10)-milk serum samples from infected mammary glands of goats from day 2 to 10 post-infusion, 11)-molecular weight marker, 12)-bacterially produced hGH. (B) Time-course of hGH expression in goats infused with different doses of AdhGH.

in both of them the hEPO expression showed a drastic drop reaching below 10 µg/mL by day eight. Independently of the expression time these results

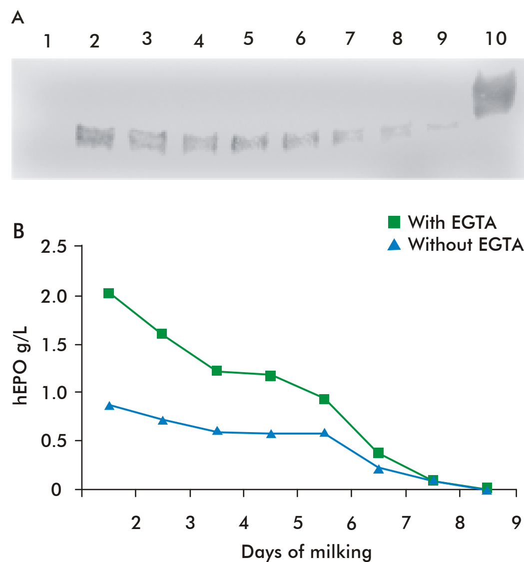

Figure 6. Expression of hEPO in the milk of goats. (A) Western blot of milk serum from a goat mammary gland infused with a solution containing 109 GTU/mL of the adenoviral vector AdhEPO. Five microliters of milk serum were loaded in each well. Lanes: 1) Milk serum from uninfected mammary glands, 2-9) Milk serum samples from infected mammary glands of goats from day 2 to 9 post-infusion, 10) CHO-hEPO. (B) Time-course of hEPO expression in goats infused with AdhEPO in the presence (▲) or absence (■) of EGTA.

demonstrate that, in fact, the formation of the tight junction in the mammary epithelium constitutes a barrier preventing the accessibility of the adenoviral vector to its receptor presumably localized on the basal side of the secretory epithelium, and through the use of a chelating agent it is possible to overcome this problem.

Discussion

Several groups have explored the direct gene transfer to the mammary glandular epithelium as a way of producing recombinant proteins in the milk of non-transgenic animals. The use of retroviral vectors [11], yielded a maximum expression of 118.6 ng/mL at the start of lactation, after which it dropped drastically below 20 ng/mL until the end of the experiment. In another study [7], receptor-mediated endocytosis was used to introduce a plasmid containing the gene of a secretable luciferase in the mammary tissue. In this experiment, the mammary gland was instilled with an insulin-polylysine-DNA complex; however, the expression levels obtained did not exceed 1 ng/mL. In a separate study, a plasmid DNA was introduced into the mammary gland of guinea pigs by complexes based on ADN/polyion interactions [6]. In this experiment, hGH was used as a secretable protein and it was expressed throughout the lactation period; nevertheless the highest expression levels of hGH detected in milk did not exceed 550 ng/mL. Fan W, *et al.* [16] reported levels of up to 1 µg/mL of the lysostaphin gene *in vivo* in the dry mammary glands of goats. Although higher than in previous reports, these expression levels were noticeably lower than what we show here. Fan and coworkers used an adenoviral vector and the same animal model as we did, but they infused only 1 mL of the viral solution into dry mammary glands, which seems to be the main reason for their low expression levels. In contrast to these earlier studies, we were able to reproducibly obtain hGH and hEPO in milk at levels above 1 mg/mL in both mice and goats, which exceeds in more than one thousand time that obtained in any of the previous reports.

Here we used an adenoviral vector based on human serotype 5 and devoid of the E1, E3 regions. This vector belongs to the early generation of adenoviral vectors for which a wealth of preclinical and clinical experiences have documented short-term expression and immune mediated destruction of transduced cells, due to the expression of viral proteins encoded in the remaining viral genome [20]. The short-term expression mediated by ΔE1ΔE3 adenoviral vectors may be a drawback when large scale biotechnological processes are require. In this regard, the use of helper-virus independent E1/E3/E4 deleted adenoviral vector seems to be a promising alternative; these vectors have provided a long term expression in gene therapy assays [27]. For such vectors, immunological response against a circulating antigen may be avoided by restricting antigen expression to the parenchymal cells of the organ involved [28] and in the absence of an immunological response against the transgene product, the main determinant of persistence of transgene expression is not an immunological response against viral epitopes but the episomal persistence of the recombinant adenoviral transgene product per se [27, 29, 30]. One of the determinants of episomal persistence is the expression cassette [27, 29]. Decrease of transgene expression of less than 20 % in the first 6 months have been achieved with these vectors [30].

Although awaiting further improvements, there is no doubt that the possibility of expressing high levels of recombinant proteins in milk through the use of adenoviral vectors offers noticeable advantages over traditional transgenesis. First, the method is simple and can be simultaneously applied to a large groups of animals, which would allow us to adjust, in a short time, the production capacity to the specific need of a given protein. The method is very flexible, allowing for the simultaneous transfer of multiple genes to attain immediate expression in the milk of multiple recombinant proteins. This fact is particularly interesting when multimeric proteins such as antibodies or protein complexes such as those formed by coagulation factor VIII and the Von Willebrand factor are desired. The expression of high levels of hEPO in the milk of mice and goats demonstrates the feasibility of the system to express proteins that due to its intrinsic toxicity it can not be expressed in transgenic animals. The high degree of structural conservation of erythropoietin and its receptor across species, makes this protein an especially difficult growth factor to be produced in the milk of transgenic animals. The ectopic expression of even small amounts of hEPO and its later release into the blood stream causes severe polycythemia in these animals with a lethal effect in the high expressing founders.

Another outstandingfeature of the technology relates to the mammary gland anatomy that allows for the exposure of the secretory epithelium to the infection by adenoviral vectors instillated through the teat channel. In this way, the epithelial cells covering the alveolar lumen constitutes the main target for infection; this fact opens the opportunity for the use of constitutive promoters such as CMV for the targeting expression restricted to the alveolar epithelium. The use of constitutive promoters, undoubtedly simplifies vector design, although this does not preclude the use of tissue specific promoters for special applications.

As a complement to transgenesis, the direct transduction of the mammary gland with adenoviral vectors could be a very useful tool to study the power of mammary-specific promoters to avoid variables associated to the position effects or to the copy numbers. The method may be also used to assess the functionality of gene constructs prior to the generation of a transgenic animal, and may be a viable approach to study the physical, chemical and biological characteristics of recombinant proteins expressed in milk of different species at the same time.

In summary, here we found that adenoviral vectors are able to successfully infect the mammary glandular epithelium and to promote the secretion of high amounts of a recombinant protein in the milk of non transgenic animals. This finding may constitute a platform for the development of new biotechnological processes directed to the production of recombinant complex proteins in milk. Ethical, regulatory and biosafety aspects regarding the use of the adenoviral vector in live animals must still be solved, although the innocuousness of such a vector in gene therapy trials seems to offer a promising future for this approach.

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