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High-Level Expression of Recombinant Human Nerve Growth Factor Beta in Milk of Nontransgenic Rabbits

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The technology for the large-scale production of therapeutic recombinant proteins remains a challenge in the biopharmaceutical industry. In this study, we reported a nontransgenic approach to producing a large quantity of human nerve growth factor beta (hNGF-β) in rabbit milk by employing a recombinant adenoviral expression system. After directly instilling hNGF-β recombinant adenoviruses into rabbit mammary glands, a polypeptide with a molecular weight of 13.2 kDa was detected in rabbit milk. The maximal expression level of hNGF-β reached 346 μg/ml. The biological activity of recombinant hNGF-β was confirmed using PC12 cells and cultures of dorsal root ganglion neurons from chicken embryos. Our data suggest that instilling recombinant adenovirus directly into the mammary gland of mammals is an efficient approach to producing a large quantity of hNGF-β.

[**Key words:** nerve growth factor, human nerve growth factor beta, adenovirus, recombinant protein, mammary gland, milk]

The nerve growth factor (NGF), the first member of the protein family termed neurotrophins, is one of the earliest cellular growth regulators discovered. Many research studies have shown that NGF is essential to the survival, growth and differentiation of central and peripheral nerves (1–6). It could also accelerate wound healing (7, 8) and restrain tumor cells (9). Some studies have suggested that NGF could improve cognitive function in impaired, aged or cholinergic lesion models (10–14). Furthermore, NGF clinical use has potential in repairing or regrowing damaged nerves, for preventing and treating retrograde neurological diseases such as Alzheimer's disease and for promoting the differentiation of neuroblasts (15–18). Recently, it has also been reported for ulcer treatment (19–23). However, the large-scale production of NGF recombinant protein still remains a challenge.

The direct purification of NGF from animal tissues is inefficient, whereas the mass production of NGF by a genetic approach has high potential. For more than a decade, several attempts have been made to produce large amounts of recombinant human nerve growth factor beta (hNGF-β) from *Escherichia coli* (24, 25), yeast (26), insect (27) and mammalian (28–30) cells. Significant progress has been achieved in increasing the yields of recombinant proteins synthesized using a mammalian cell system. However, this system could not supply sufficient NGF for clinical use. Although the use of transgenic animals could be considered as another method for the large-scale production of hNGF-β (31) in terms of overall yield (32), the long time and high expense for constructing a transgenic system make the large-scale production of recombinant hNGF-β uneconomical.

In recent years, a new approach to expressing exogenous genes in secretory mammary epithelial cells has been proposed. A target protein could be obtained from milk by employing a recombinant adenovirus directly transfected into the mammary gland. This has been proven to be the most effective versatile alternative (33). Such an approach has been shown to promote the entry of incorporation DNA into the mammary gland and to result in high expression levels of encoded genes. Although some attempts have been made to obtain target proteins using this approach (34–41), only a few have successfully obtained the high expression levels of target proteins in milk of different animals (38–41). As suggested in previous reports, the successful expression of a recombinant protein using this method relies on many factors, including the physiological and biological properties of the recombinant protein itself, the promoter driving the recombinant protein expression *in vivo*, and the animals used to express the recombinant protein. To our best knowledge, hNGF-β has not been successfully produced in milk of nontransgenic animals.

In this study, we used rabbits as animal models to produce hNGF-β in the mammary gland. The replication-defective recombinant adenovirus encoding hNGF-β gene was constructed and transfected into rabbit mammary glands in lac-

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tation. The expression level of recombinant hNGF-β in milk was significantly higher than those that have been reported so far using other expression systems (24–30, 32).

MATERIALS AND METHODS

RNA extraction, cDNA synthesis, and PCR amplification of hNGF-β A human embryo heart was obtained legally. Total RNA was extracted from the human embryo heart with Trizol reagent according to the protocol described by the supplier (Promega, Beijing, China). The quantification and integrity of the RNA were checked by measuring UV absorbance at 260 and 280 nm and by electrophoretic analysis, respectively. Ten microliters of reverse transcription reaction mixture containing 5 μg of total RNA, 500 μM dNTPs, 0.2 μM each of the primers and DEPC-treated water was incubated at 65°C for 5 min, and then placed on ice for 1 min. After that, reverse transcription was carried out in the presence of 1 μl of superscript III reverse transcriptase (200 U/μl), 1 μl of RNase inhibitor (40 U/μl), 2 μl of 10×buffer, 4 μl of $MgCl_2$ (25 mM) and 2 μl of DTT (0.1 M) at 50°C for 50 min. Subsequently, the reaction was terminated at 85°C for 5 min. The reverse transcription product was kept at −20°C until use. PCR amplification was performed on a PTC-200 Peltier Thermal Cycler (MJ Research, Munich, Germany). One microliter of the reverse transcription product was subjected to amplification in 50 μl of reaction mixture containing 1.5 U of KOD Plus DNA polymerase, 5 μl of 10×PCR buffer, 100 μM dNTP, and 25 pM each of primers P1 and P2 (see below). The PCR cycle was as follows: initial denaturation at 95°C for 5 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 90 s; and a final extension at 72°C for 7 min. The complete hNGF-β coding region was amplified with the following primer pair: the forward primer P1: 5′-GTggtaccGCATAGCGTAATGTCCAT-3′ (corresponding to positions 152–177 of the NGF-β mRNA [GenBank accession no. NM_002506]) with an introduced *Kpn*I recognition sequence (underlined in the primer); and the reverse primer P2: 5′-GTctcgagTC GGCAGGTCAGGC-3′ (positions 890–911) with an introduced *Xho*I recognition sequence (underlined in the primer). All the primers used in the study were synthesized in SBS Genetech (Beijing, China). The PCR product was purified and cloned into pcDNA3.1 (+) vector (Invitrogen, Shanghai, China) followed by sequencing.

Construction of recombinant adenoviral vectors All standard recombinant DNA protocols were followed as described previously (42, 43). The replication defective adenovirus vector AdhNGF was generated using the AdEasy adenoviral vector system (44). First, the fragment containing hNGF-β cDNA was digested with *Kpn*I and *Xho*I endonucleases. The digested fragment of 760 bp was then cloned into the corresponding sites of the pShuttle-CMV plasmid. The resulting plasmid was designated pshNGF (8125 bp) and used in the next reaction. At the same time, a DNA fragment of the green fluorescent protein (GFP) gene was excised from the p3.1egfp vector (constructed by our institution) using *Not*I and *Pme*I. Following gel purification, a DNA fragment of 753 bp was cloned into the *Sma*I and *Not*I sites in the vector pIRES (Takara, Dalian, China). The resulting plasmid was designated pIR-G (6820 bp). Then a DNA fragment of 1308 bp including IRES and GFP was excised from pIR-G by *Xho*I and *Eco*RV. After gel purification, the 1308-bp fragment was cloned into pshNGF, which was digested with *Xho*I and *Eco*RV and purified before use. The resulting vector was designated pSh-hNGF-GFP.

The plasmid pSh-hNGF-GFP was digested with *Pme*I. The product of digestion and an Ad-Easy vector (Stratagene, La Jolla, CA, USA) containing the adenovirus type 5 genome deleted E1 and E3 regions were simultaneously transformed in ultracompetent BJ5183 bacterial cells to yield an Ad-hNGF recombinant adenovirus vector. The Ad-hNGF vector was linearized with *Pac*I and purified using a DNA Purification kit (BioDev-Tech, Beijing, China) according to the manufacturer's instructions. The final linearized Ad-hNGF was stored at −70°C until use.

Preparation of viral stocks Primary viral stocks were prepared by transiently transfecting the linearized Ad-hNGF into host HEK-293 cells. After further amplification in HEK-293 cells, the adenoviral stocks were collected and purified using an Adeno-X Virus Purification kit (Takara, Dalian, China). The resultant Ad-hNGF vector in the adenovirus contained both the genes of GFP and hNGF-β under the control of the cytomegalovirus (CMV) promoter. Therefore, the adenoviral stocks could be titrated by the GFP expression in semi-confluent HEK-293 cells. After the titer of each viral stock was determined from the GFP expression in semi-confluent HEK-293 cells, the recombinant adenovirus stock was stored at −70°C until use.

***In vitro* expression** Rabbit mammary epithelial (RME) cells were counted using a cytometer and seeded in 100-mm dishes at a density of about 2.5×10^5 cells/cm^2 in DMEM supplemented with 10% FBS, 10 ng/ml epidermal growth factor, 10 μg/ml insulin and 1% penicillin–streptomycin. After the cells grew up to about 70% confluences, the medium was replaced with fresh DMEM without serum as the harvest medium. The cells were infected with the recombinant adenovirus at multiplicities of infection (MOIs) of 10, 20, 40, 80 and 160. Twenty-four or 72 h later, the medium was harvested and centrifuged at 12,000 rpm for 10 min. The supernatant was collected and assayed for recombinant hNGF-β content.

Expression of hNGF-β in milk Ten adult female rabbits (4.0±0.5 kg) were maintained for adenoviral infusion and divided randomly into five groups in the animal facility of the Department of Biological Engineering of Yanshan University. The adenoviral infusion was performed as described elsewhere (39, 41, 45). For all mammary infusions and sample collections, the teats were routinely sterilized with 70% alcohol and iodine to prevent mammary gland infection. The rabbits were anesthetized for adenovirus infusion by intraperitoneal injection of 0.6 ml of Sumianxin (Veterinary Institute of Jilin University, China). The four right ventral mammary glands were infused with 400 μl of PBS containing the desired amount of the recombinant adenoviral vector. The four left ventral mammary glands of one of the two rabbits in each group were infused with the same volume of PBS containing the desired amount of a vacant adenoviral vector and taken as the control; the four left ventral mammary glands of the other rabbits were infused with the same volume of PBS and taken as the negative control.

After infusion for 24 h, all the glands were milked at 6:00 pm on the indicated days. The milk samples from the experimental, control or negative control glands of each rabbit were pooled within the group for analysis. The collected milk was immediately centrifuged at 12,000×*g* for 20 min at 4°C. The supernatant of the milk was separated from the layer as completely as possible, and then diluted with distilled water of five volumes. The diluted supernatant of the milk was adjusted to pH 4.5 by adding 10% acetic acid. After 3-h incubation at room temperature, the precipitated protein was removed by centrifugation at 17,000×*g* for 30 min. The supernatant was immediately neutralized with 1 M NaOH and clarified by sterile filtration (0.22 μm). The clear filtrate was designated as the milk serum and stored at −70°C until assay.

rhNGF-β detection For qualitative assay, the supernatant of the cell culture and the milk serum of the rabbits were separated on SDS-polyacrylamide gel electrophoresis (PAGE) in 12% gels, and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) on a Trans-Blot and Semi-Dry Electrophoretic Transfer Cell (Biometra, Goettingen, Germany). The membranes were subjected to Western blot analysis with an anti-hNGF-β monoclonal antibody (R&D Systems, Minneapolis, MN, USA) as the primary antibody and goat anti-mouse IgG con-