Human nerve growth factor beta (hNGF-β): mammary gland specific expression and production in transgenic rabbits

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Abstract Transgenic rabbits carrying gene constructs encoding human nerve growth factor beta (hNGF-β) cDNA were generated. Expression of hNGF-β mRNA was restricted to the mammary gland of lactating rabbits. Western Blot analysis revealed a polypeptide of 13.2 kDa in the milk of transgenic animals. hNGF-β was purified from the milk by a two-step chromatographic procedure. Electrospray mass spectroscopy analysis of purified hNGF-β depicted a molecular weight of 13 261 Da per subunit. The biological activity of the hNGF-β was tested using PC12W2 cells and cultures of dorsal root ganglion neurons from chicken embryos. Crude defatted milk from transgenic animals and purified hNGF-β demonstrated full biological activity when compared to commercial recombinant hNGF-β.

Key words: Human nerve growth factor beta; Bovine αs_1-casein; Transgenic rabbit; PC12W2 cell; Dorsal root ganglion neuron

1. Introduction

Nerve growth factor (NGF), the founder member of a protein family termed neurotrophins, is known to promote the survival and differentiation of primary sensory neurons, sympathetic neurons and cholinergic neurons of the basal forebrain [1–4]. Several attempts have been undertaken to produce a large quantity of recombinant hNGF from *Escherichia coli* [5], yeast [6], insect cells [7] and mammalian cell cultures [8,9]. Preclinical studies using recombinant hNGF-β to treat neuronal dysfunction of the central and peripheral nervous systems [10,11] and clinical trials including the treatment of HIV-related peripheral neuropathy carried out by Genentech Incorporated are potential indications for the need of a large quantity of biologically active hNGF-β. In the last 10 years tremendous progress has been made in increasing yields of recombinant proteins synthesized by mammalian culture systems. However, for large-scale production, transgenic technologies can be considered according to their overall yield [12]. Here, we describe a mammary gland specific expression of hNGF-β cDNA under the control of the regulatory elements of the bovine αs_1-casein gene and the secretion of functional hNGF-β in the milk of transgenic rabbits.

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2. Materials and methods

2.1. Generation and analysis of hNGF-β transgenic rabbits

Transgenic rabbits were generated by microinjection of p180 constructs (Fig. 1) into fertilized oocytes as described in [13–15]. For the detection of transgenic animals, PCR analysis of genomic DNA extracted from ear or tail biopsies using Oligo248 and Oligo249 was performed (see Fig. 1).

2.2. Analysis of transgenic protein: SDS-PAGE and Western blot

Defatted milk from lactating transgenic and non-transgenic rabbits was diluted 1:10 with 10 mM Tris-HCl pH8.0/10 mM $CaCl_2$. Diluted milk (2 μl) was mixed with 2×sample buffer (SDS reducing buffer), heated at 95°C for 5 min and fractionated on a 15% SDS-PAGE according to Laemmli [16]. The transfer of proteins to nitrocellulose membranes (Immobilon NC pure, Millipore, Austria) was performed according to standard protocols [17]. Detection of hNGF-β was carried out with goat polyclonal anti-hNGF antibodies (R&D Systems, UK), secondary rabbit anti-goat IgG peroxidase conjugate (Sigma Immuno Chemical, USA) and chemiluminescence (ECL, Amersham, UK) according to the manufacturers' protocols. Recombinant hNGF-β synthesized in the mouse myeloma cell line NSO (R&D Systems, UK) was applied as a standard control.

2.3. Analysis of transgenic mRNA expression: RT-PCR

Total RNA was extracted with Trizol (Gibco BRL, Life Technologies, Austria) from mammary gland biopsies during lactation. First strand cDNA was synthesized using oligodT in the presence of M-MLV reverse transcriptase (Gibco BRL, Life Technologies, Austria) according to standard protocols [17]. Two p180 specific oligonucleotide primers (oligo4 and oligo5) were designed to screen the expression of hNGF-β (see Fig. 1). For the amplification control, the expression of rabbit α-actin was assayed using the following oligonucleotides: 5′-TGTGACATCGACATCAGGAAGG-3′ and 5′-TAGGTAATGAGTCAGAGCTTTGG-3′ (nt 917–938 and nt 1277–1299, GenBank accession number X60732).

2.4. Cell culture and bioassays

The biological activity of NGF (enhancing cell survival and inducing neurite outgrowth) was determined using PC12W2 cells cultured as described in [18,19]. To test for neurite outgrowth, PC12W2 cells ($0.7–1\times10^4$ cells/well) were maintained on collagen coated 6-well tissue culture plates (Falcon, Becton Dickinson) in the medium containing 2% serum (1% inactivated horse serum and 1% inactivated fetal calf serum). Defatted milk from transgenic and non-transgenic (control) rabbits was diluted with RPMI 1640 containing antibiotics. Neurite outgrowth was investigated with dose and time dependent experiments. Diluted milk containing rhNGF-β in doses of 1, 2, 5, 10, 25 and 50 ng/ml medium (dose experiment) and a single dose of 50 ng/ml medium (time experiment) was added to the cells and compared to the same concentrations of commercial recombinant hNGF-β (R&D Systems) likewise diluted with RPMI 1640. Untreated cells and cells treated with non-transgenic milk diluted with RPMI 1640 were used as standard controls. Fresh medium with or without additive was added on day 3. On day 6 (dose dependent neurite outgrowth) and days 2, 4, and 6 (time dependent neurite outgrowth) the cells were examined and five microscopic fields were randomly photographed

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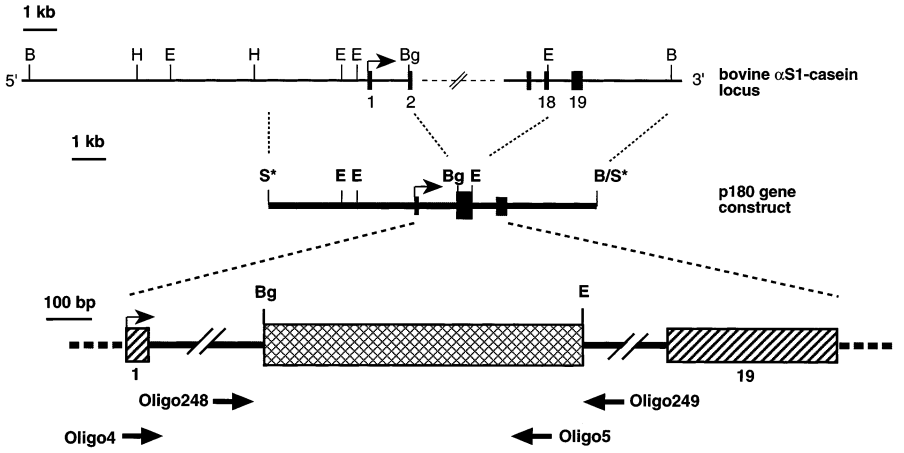

Fig. 1. Mammary gland specific gene construct p180 (7.2 kb) and the position of primers used for PCR analysis. The structure of this expression cassette has been described in detail [22]. Here, a 0.717 kb fragment (from Met^{-121} to Arg^{+118} [38]) of the cDNA encoding the human pre-pro-NGF-β was inserted as a fusion gene into this expression cassette under the control of bovine αs_1-casein promotor. A 5′ UTR (2.9 kb) consisting of exon 1 (52 bp, left hatched box) and part of intron 1 and 3′ UTR (3.6 kb) containing exon 19 (384 bp, right hatched box) and part of intron 18 are shown. The fusion gene (cross-hatched) comprises bovine αs_1-casein signal peptide sequence (57 bp), the sequence of hNGF-β cDNA (717 bp) and a short part of bovine αs_1-casein 3′ UTR (12 bp). The positions of oligonucleotide primers for detection of transgenic animals (p180 rabbits) are indicated: oligo248 (5′-GATGCTTCTCTATTCCTCTG-3′, nt 3266–3285 [39], accession number X59856) and oligo249 (5′-GACCATAACTGTGGAGTCCC-3′, nt 18 442–18 461 [39], accession number X59856). The position of synthetic hNGF-β primers used to characterize the expression of hNGF-β are also shown as follows: oligo4 (5′-GATCATCAACCCAGCTTGCTC-3′, nt 2140–2161 (exon 1) [39], accession number X59856) and Oligo5 (5′-TGCCATCCATGGTCAGCGCCT-3′, nt 795–815, cDNA sequence of hNGF-β EMBL [40], accession number X52599). The predicted amplification product is 692 bp comprising 46 bp of exon 1 at the 5′ αs_1-casein sequence and 646 bp of hNGF-β cDNA. B, *Bam*HI; H, *Hin*dIII; E, *Eco*RI; Bg, *Bgl*II, S*, *Sal*I site of the 5′ derived from the cloning vector.

using a Nikon Diaphot 300 microscope. Neurite bearing cells were counted on the negative films. The bioassays for each concentration of rhNGF-β as well as for controls were done in triplicate.

The bioassay with primary cultured neuronal cells was performed as described in [20]. Briefly, dorsal root ganglion neurons were extracted from chicken embryos, cultured in F14 medium [1] with 10% horse serum and antibiotics and then seeded onto poly-ornithine/laminin coated microtiter plates in the presence or absence of known concentrations of rhNGF standard (preparation from *E. coli*, Boehringer Mannheim, Penzberg, Germany) or purified (see below) rhNGF-β from transgenic rabbits. Neuronal survival was assessed after 48 h by counting the number of surviving neurons with neurite elongation under a phase contrast microscope.

2.5. Purification of rhNGF-β from milk of transgenic rabbits

The skim milk was adjusted to pH 4.5 by addition of 10% acetic acid and diluted with distilled water (2 vol.). After 3 h incubation at room temperature the precipitated protein was removed by centrifugation at $17\,000 \times g$ for 30 min. The supernatant (whey acid) was immediately neutralized with 1 M NaOH and clarified by sterile filtration (0.20 μm). The clear filtrate was loaded onto a HiTrap SP column (Pharmacia Biotech, Freiburg, Germany) which was equilibrated with 100 mM sodium acetate, pH 6.5. After washing with equilibration buffer, the retained proteins were eluted with a NaCl gradient to 1 M. The rhNGF-β containing fractions were pooled and purified to homogeneity by chromatography on HA Ultrogel (Biosepra, Schermbeck, Germany) under essentially the same conditions as before. Samples were analyzed by SDS-PAGE using the Laemmli buffer system [16] and by RP-HPLC on a POROS R1/H (PerSeptive Biosystems, Wiesbaden, Germany) with a linear gradient ranging from 5 to 80% acetonitrile in 0.1% trifluoroacetic acid. Concentration of pure rhNGF-β was determined by UV spectroscopy using $A^{1\%}$=1.50 at 280 nm [21].

2.6. Electrospray mass spectroscopy

The RP-HPLC fractions were applied to the mass spectrometer (API 100 from PE Sciex, Canada) without further preparation. The mass analyzer was scanned from 1400 to 2400 amu with a step width of 0.2 amu. The orifice voltage and the flow rate of analyte solution were set at 80 V and 5 μl/min, respectively. The molecular mass spectrum was obtained by deconvoluting the mass/charge spectrum with the software Biospec Reconstruct from PE Sciex. The relative error of mass determination was 0.01%.

3. Results

3.1. Generation of p180 rabbit lines

Four established lines #2047, #2048, #2049 and #5432 were used for hNGF-β production. F0 females #2047 and #2049 and F1 individuals for all four lines were studied at the transcriptional as well as the translational level for hNGF-β (Table 1).

3.2. Secretion of hNGF-β into the milk of transgenic rabbits

A polypeptide of 13.2 kDa corresponding to the molecular

Table 1
Established transgenic lines for the production of hNGF-β

Line	F0	Expression analysis			F1 (females)	Expression analysis		
		RNA	Protein	Ectopic		RNA	Protein	Ectopic
1	2047 (f)	+	+	−	7299	+	+	−
2	2048 (m)	ND	ND	ND	2211	+	+	ND
3	2049 (f)	+	+	−	7255	+	+	−
4	5432 (m)	ND	ND	ND	5749	+	+	ND
					5756	+	+	ND

ND, not determined; f, female; m, male.

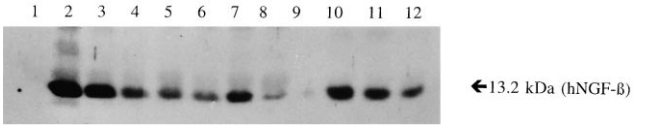

Fig. 2. Protein analysis/Western blotting analysis of milk from transgenic and non-transgenic rabbits. The samples were processed and immunodetected as described in Section 2. Commercial recombinant from mouse myeloma cell line NSO (R&D Systems, UK) served as positive control. Lane 1, non-transgenic rabbit; lane 2, 2211, F1 #2048; lane 3, 5749, F1 #5432; lane 4, 5756, F1# 5432; lane 5, 7255, F1 #2049; lane 6, 2049, F0 #2049; lane 7, 7299, F1 #2047; lane 8, 2047, F0 #2047; lane 9, no sample; lanes 10–12, 50 ng, 25 ng, 12.5 ng of commercial rhNGF mixed with non-transgenic milk.

weight of hNGF-β was detected by Western blot in the milk of the two F0 females, their offspring and the offspring of the male founders. The concentration of hNGF-β in the milk of transgenic rabbits was determined by comparison of Western blot signals of transgenic milk samples with known concentrations of commercial recombinant hNGF-β mixed with non-transgenic milk. The level of hNGF-β in the milk varied in the different transgenic lines from 50 to 250 mg/l (Fig. 2). The estimated concentrations were confirmed by a bioassay (see below).

3.3. RNA expression analysis of hNGF-β

Expression of hNGF-β mRNA was found in the mammary gland of all examined lactating transgenic rabbits as shown by RT-PCR. The PCR products showed the expected size of 692 bp. A lactating non-transgenic rabbit female was used as a control and did not show any expression of NGF-β (Fig. 3A). Different tissues (muscle, heart, lung, liver, pancreas, spleen, kidney, ovary, parotid gland, cerebrum, cerebellum, submaxillary gland, and mammary gland) from transgenic rabbits (2047, 7299 and 7255) secreting 50, 50 and 70 mg/l hNGF-β

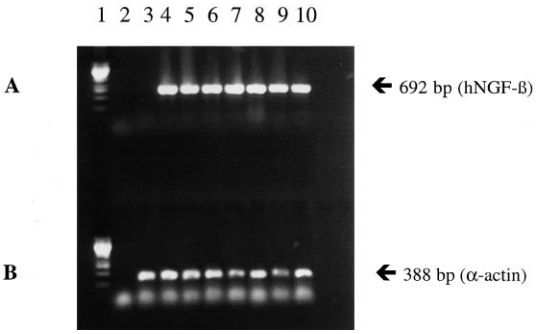

Fig. 3. RNA analysis/RT-PCR. Total RNA was extracted from lactating non-transgenic and transgenic rabbits and RT-PCRs were performed as described in Section 2. Amplification products are shown on 1.5% agarose gel stained with dimidium bromide. A: Lane 1, DNA ladder; lane 2, non-template control; lane 3, non-transgenic rabbit; lane 4, 2211, F1 #2048; lane 5, 5749, F1 #5432; lane 6, 5756, F1 #5432; lane 7, 7255, F1 #2049; lane 8, 7299, F1 #2047; lane 9, 2049, F0 #2049; lane 10, 2047, F0 #2047. B: Expression analysis of rabbit α-actin.

in their milk, respectively, were investigated. The expression of hNGF-β was restricted to the mammary gland of lactating transgenic rabbits (Fig. 4). Furthermore, the expression analysis of 2047 (F0) and its F1 offspring (7299) was identical indicating that the lack of ectopic expression in the F0 individual is not due to transgene mosaicism. The RT-PCR was verified by amplification of rabbit α-actin and the expected fragment with 388 bp is shown (Figs. 3B and 4).

3.4. Bioassays

On day 6, cells treated with milk containing 50 ng/ml hNGF-β exhibited substantial neurite outgrowth, whereas most cells treated with milk from non-transgenic rabbits as well as untreated cells died (Fig. 5). Furthermore, neurite outgrowth increased with both dosage and time and the biolog-

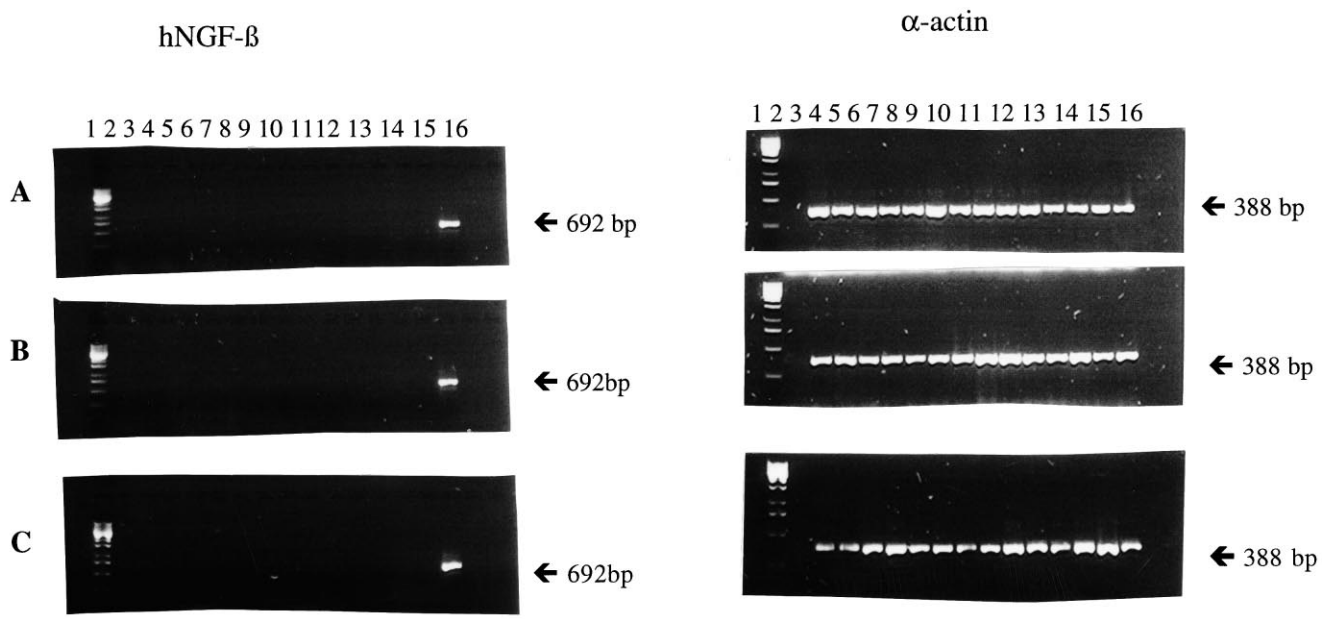

Fig. 4. Ectopic expression analysis/RT-PCR. RNA isolated from different tissues, RT-PCR and gel electrophoresis were performed as previously described for RT-PCR. Lane 1, DNA ladder; lane 2, non-template control; lane 3, muscle; lane 4, heart; lane 5, lung; lane 6, liver; lane 7, pancreas; lane 8, spleen; lane 9, kidney; lane 10, ovary; lane 11, parotid gland; lane 12, cerebellum; lane 13, cerebrum; lane 14, submaxillary gland; lane 15, mammary gland of non-transgenic rabbit; lane 16, mammary gland of p180 transgenic rabbit. A: 7255, F1 #2049; B: 7299, F1 #2047; C: 2047, F0 #2047.

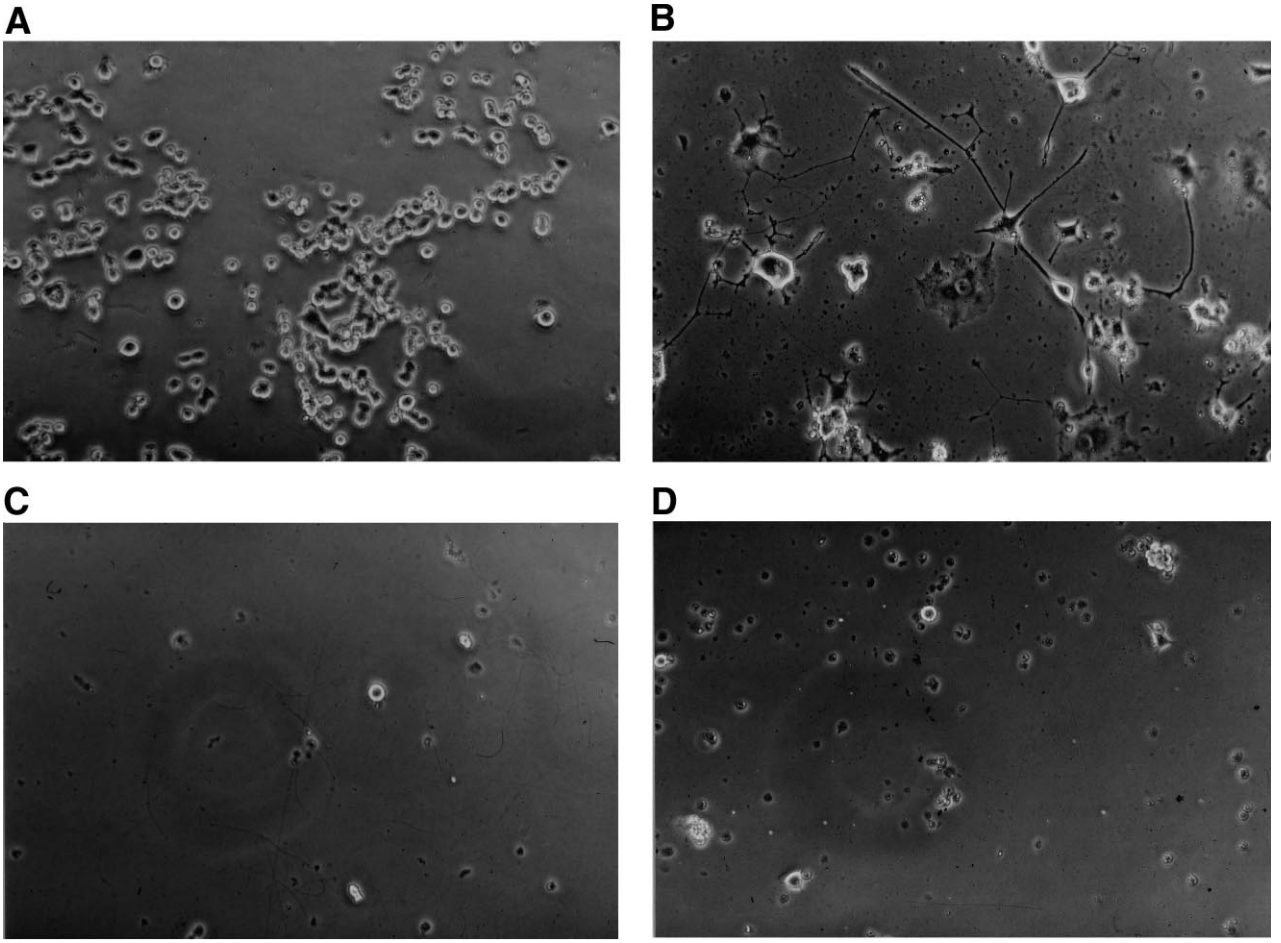

Fig. 5. Morphology of PC12W2 cells. PC12W2 cells were cultured as described in Section 2. For the bioassay, cells were washed, maintained in RPMI/2% serum (1% FCS and 1% horse serum) and treated on days 1 and 3 as described in Section 2. Morphology of PC12W2 cells cultured on day 6 in RPMI/15% serum (A); in RPMI/2% serum in the presence of diluted transgenic milk containing 50 ng/ml medium (B); in RPMI/2% serum in the presence of the milk from non-transgenic rabbit (C); in RPMI/2% serum (D). Cells were examined under a microscope and photographed (12×).

ical activity of our recombinant hNGF-β was equivalent to that exhibited by the commercial recombinant hNGF-β. The results show increased neurite outgrowth in both experiments for rhNGF-β and commercial rhNGF-β (Fig. 6A,B). In addition, the biological activity of purified factor from the milk was assessed using primary tissue culture (dorsal root ganglion neurons) from chicken embryos. Again, the rhNGF-β from transgenic rabbits (#2047) displayed the same biological activity as a standard preparation from *E. coli* (Boehringer Mannheim, Penzberg, Germany). The neurotrophic activity was expressed as one half of the maximal stimulation index (EC_{50}), e.g. 100 pg/ml for rhNGF-β (data not shown).

3.5. Purification and mass spectroscopy

The rhNGF-β purified from the milk of transgenic rabbits by the two-step chromatographic procedure was shown to be >95% pure by SDS-PAGE and RP-HPLC (data not shown). Electrospray mass spectroscopy analyses of purified hNGF-β depicts the mass/charge as well as the derived molecular mass spectrum (Fig. 7). In addition to molecules with a mass of 13 261.3 amu (expected monomeric molecular weight of rhNGF-β: 13 261.1 amu), molecules with a monomeric molecular mass of 13 104.3 amu were detected. This additional molecular species corresponds to rhNGF-β molecules lacking the C-terminal arginine (theoretical molecular mass: 13 104.9 amu). Its abundance exceeds the yield of intact rhNGF-β by a factor of two. In samples purified with omission of the acidifying step, the truncated species was not detectable (data not shown). However, preparation without this step is likely to be hampered by milk components retaining the recombinant protein. Purified rhNGF-β from the milk of transgenic rabbits has the same biological activity as a standard preparation from *E. coli*.

4. Discussion

We have used a mammary gland specific expression cassette containing the cDNA encoding the prepro-hNGF-β under the control of bovine αs_1-casein regulatory sequences to generate transgenic rabbits. Four lines were established and studied at the level of transcription and translation. Stable, lactation stage and strictly mammary gland specific production of hNGF-β was found in the milk of all analyzed transgenic rabbits. The production rate varied in the different lines from 50 to 250 mg/l. Several laboratories have reported the production of biologically active hNGF, e.g. from mammalian cell culture 4 mg/l, 2.5 mg/l [8,9], *E. coli* 2.7 mg/l [5], yeast 1 mg/l [6] and insect cells 20 mg/l [7]. Although our produc-

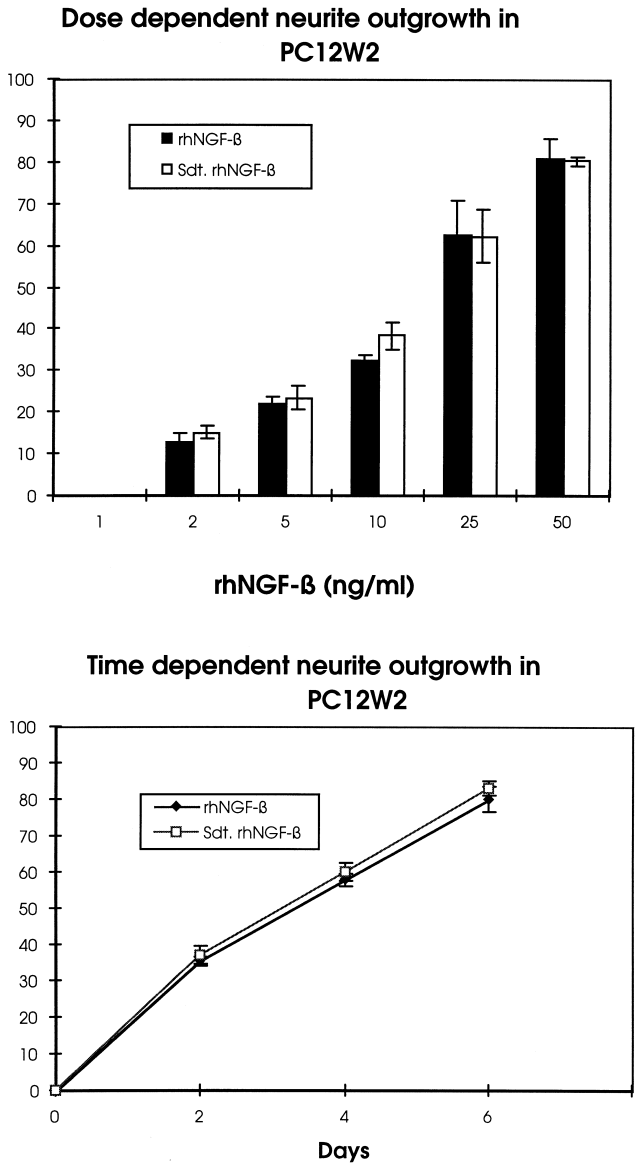

Fig. 6. Dose and time dependent neurite outgrowth in PC12W2 cells. Cells were maintained in medium containing 2% serum and rhNGF-β or commercial rhNGF-β was added as described. A: On day 6 for dose dependent neurite outgrowth in response to diluted milk containing 1, 2, 5, 10, 25 and 50 ng/ml of rhNGF-β (■) and commercial rhNGF-β (Std. rhNGF-β) diluted with RPMI (□). B: Time dependent neurite outgrowth in response to diluted milk containing 50 ng/ml on days 2, 4 and 6 for rhNGF-β (♦) and commercial rhNGF-β (50 ng/ml) (□). Five random microscopic fields (magnification is 10×) were photographed and neurite bearing cells were counted on the negative films. Neurite bearing cells were scored as cells with neurites longer than the cell body. Percent neurite outgrowth was calculated as the number of cells with neurites×100/total number of cells counted. Experiments were done in triplicate. Error bars represent S.E.M. ($n=3$).

tion rate is much higher than most reported to date, it is nonetheless lower than expected. Using the same expression cassette we have recovered 0.1–1 g/l rhIGF-1 [22,23]. Varied production rates in the milk of transgenic mice were reported using bovine αs_1-casein regulatory sequences [24]. Variation in the expression of the gene constructs in transgenic animals seems to be a common phenomenon which probably depends on the regulatory elements of promoters, the sequences of the gene of interest (genomic sequences or cDNA), the integration sites and the number of integrated copies of the gene of interest (for detailed discussion and references see [23]).

The established cell line PC12W2 and primary dorsal root ganglion neurons from chicken embryos were used to assay the biological activity of non-purified and purified rhNGF-β, respectively. Both systems demonstrated clearly that rhNGF-β is biologically active in crude milk as well as purified from the milk. The biological activity of rhNGF-β in each system was comparable to that shown by the standard control rhNGF-β. Rat pheochromocytoma cells (PC12) are a well established cell line system to test survival and neurite outgrowth in the presence of NGF [25,18,19]. We show that 6 days of culture 1 ng/ml of rhNGF-β or commercial rhNGF-β is required for survival but not sufficient for differentiation. In contrast, Altar et al. [26] and Schmelzer et al. [9] showed neurite outgrowth in PC12 cells at 39–52 pg/ml and 100–200 pg/ml rhNGF, respectively. We have observed massive cell death for untreated cells or cells treated with milk from non-transgenic rabbits. Glassman et al. [27] reported that more than 80% of native PC12 cells survived 1 week in 3% serum without NGF. In PC12W2 cells, neurite outgrowth starts at 2 ng/ml with maximal induction of cell differentiation at 50 ng/ml medium on day 6 for rhNGF-β as well as for the standard commercial rhNGF-β. The discrepancies in the bioassays may be due to the cell line used or the culturing conditions of the cells. Serum or plasma fractions from p180 rabbits did not

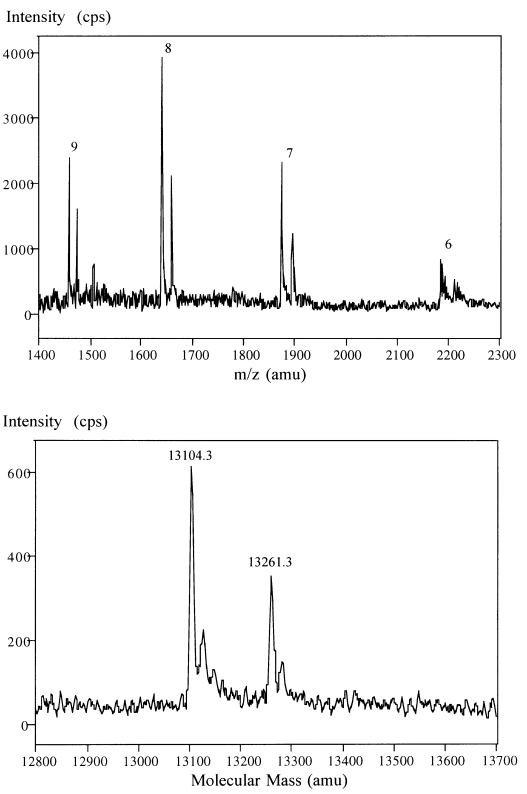

Fig. 7. Electrospray mass spectroscopy of rhNGF-β prepared from p180 rabbit (#2047) milk. Top: mass/charge (m/z) spectrum, the numbers above the peaks correspond to the numbers of protons bound to analyte molecules. Bottom: molecular mass spectrum obtained by deconvolution of the m/z spectrum. The peaks on the high mass side of the main peaks are due to the formation of sodium adducts during the ionization process.

induce any neurite outgrowth in PC12W2 cells indicating the absence of ectopic transgene expression as has been confirmed by RT-PCR analyses. In addition, this suggests the absence of resorption of hNGF-β from milk to blood at these expression levels. High expression of hNGF-β mRNA was found exclusively in the mammary gland of lactating transgenic rabbits (Figs. 3 and 4). Similar results have been reported [22,28,24] using bovine αs_1-casein regulatory sequences. The transgenic rabbits did not show any transgene specific detrimental effects in their physiological or reproductive performance. In contrast, specific overexpression of NGF in transgenic mice produced morphological (hypertrophy and hyperplasia) and functional changes (hyperinnervation) of organs within which the transgene was expressed [29,30]. Our results show that the mammary gland of transgenic rabbits is a possible source of recombinant hNGF-β with the properties of native hNGF-β. Rabbit husbandry can be carried out under specific pathogen free conditions and to date no infectious agent pathogenic for humans, which might be difficult to remove by the purification procedure of the recombinant protein, has been found in rabbits. rhNGF-β was easily purified to homogeneity and exhibited the same biological activity as commercial rhNGF-β. The C-terminal truncation observed by electrospray mass spectroscopy analysis has no obvious influence on the biological activity of rhNGF as has been demonstrated previously [9,31]. However, research is in progress to avoid the cleavage of C-terminal arginine. Studies on the physiology of NGF and with rhNGF in animal models indicated the beneficial effects of exogenous NGF for the treatment of age dependent neuronal dysfunction [32–35] or peripheral neuropathic diseases [36,37] in humans.

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