A SIMPLE AND EFFICIENT METHOD FOR THE PRODUCTION OF HUMAN GLYCOSYLATED GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR USING A SEMLIKI FOREST VIRUS EXPRESSION SYSTEM

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ABSTRACT

Human glial cell line-derived neurotrophic factor (hGDNF) is a very promising protein for the treatment of Parkinson´s disease and other neurodegenerative disorders. The present work describes a quick and simple method to obtain a high amount of purified hGDNF using a mammalian cell-derived system. The method is based on the high expression level provided by a Semliki Forest virus vector and its ability to induce a strong shut-off of host-cell protein synthesis in mammalian cells. As a result, hGDNF is the only protein present in the supernatant and can be efficiently purified by a single chromatographic step. Using this system it was possible to eliminate other secreted proteins from the culture medium, like insulin-like growth factor-5, which are hard to remove using other hGDNF production methods. Purified hGDNF presents a complex glycosylation pattern typical of mammalian expression systems and is biologically active. This protocol could be extended to other secreted proteins and could be easily scaled up for industrial purposes.

Keywords: GDNF, SFV expression system, protein expression, protein purification, glycosylated recombinant protein, BHK cells.

1. INTRODUCTION

Therapeutic proteins represent the largest class of new products being developed by the biopharmaceutical industry, with US sales reaching about $46.5 billion in 2008 (Aggarwal, 2009). Currently, there are over 165 recombinant proteins approved for human use, with other 500 candidates under preclinical or clinical development (Durocher and Butler, 2009). The unique properties of proteins in terms of potency and safety have revolutionized the treatment of many diseases where other therapeutic approaches have failed (Werner et al., 2007). Given the fact that post-translational modifications affect biochemical and therapeutic properties, more than one-third of approved biopharmaceuticals are glycoproteins (Walsh and Jefferis, 2006). The need of glycosylation has made the mammalian cell–based systems the method of choice for protein production, as opposed to technically more straightforward and economically more attractive bacterial-based systems. In order to reach the market, the process of glycosylated recombinant protein production and purification should be fast, reproducible and cheap.

Glial cell line-derived neurotrophic factor (GDNF) was described as a potent neurotrophic factor for dopaminergic neurons (Lin et al., 1993). This neurotrophic property made GDNF a promising therapeutic protein for the treatment of Parkinson´s disease (PD) and other neurodegenerative disorders like amyotrophic lateral sclerosis, spinal cord injury, peripheral nerve injuries or ocular diseases (Carnicella and Ron, 2009; Ciriza et al., 2008; Cheng et al., 2002; Guzen et al., 2009; Jiang et al., 2007; Mohajeri et al., 1999; Sharma, 2006; Suzuki et al., 2008). In fact, the neuroprotective and neurorestorative effects provided by GDNF in different PD animal models led to clinical trials. Two initial open-label phase I clinical trials of PD patients based on direct GDNF infusion into the putamen yielded very promising results (Gill et al., 2003; Patel et al., 2005; Slevin et al., 2005). However, a following double-blind placebo-controlled trial did not show significant therapeutic improvement in patients

receiving GDNF (Lang et al., 2006). The appearance of antibodies against GDNF in some patients was one of the reasons to halt the clinical trial. Whereas human GDNF is a highly glycosylated protein, the protein infused into patients had been produced in *E. coli,* lacking the oligosaccharide chains found in the human protein (Sherer et al., 2006). Nevertheless, GDNF is still considered one of the most promising molecules for the treatment of PD. However, in order to reach the clinic, it would be necessary to obtain a protein as similar as possible to human endogenous GDNF (hGDNF). The aim of the present work was to develop a mammalian-based expression system to express and purify hGDNF in a simple way. An alphavirus vector derived from Semliki Forest virus (SFV) was chosen as expression system (Liljestrom and Garoff, 1991). This system is based on a self-replicating RNA vector that upon transfection into cells is translated to produce the viral replicase. This enzyme mediates both the amplification of the RNA vector and the synthesis of a subgenomic RNA from which the desired heterologous protein is translated at high levels. The fact that vector replication induces a strong shut-off of host-cell protein synthesis represents an important advantage for this system. A SFV vector able to express high levels of hGDNF in mammalian cells was constructed. The results showed accumulation of hGDNF in the supernatant of transfected cells, while production of endogenous secretory proteins was strongly inhibited. This hGDNF, which was glycosylated and biologically active, could be easily purified by a single chromatographic step. In conclusion, we have developed a simple and quick method to purify hGDNF.

2. MATERIALS AND METHODS

2.1. Recombinant proteins and immunological reagents. Recombinant hGDNF produced in bacteria was purchased from Invitrogen (Carlsbad, CA). Polyclonal antibodies specific for GDNF, insulin-like growth factor-binding protein-4 and -5 (IGFBP-4 and IGFBP-5) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and for β-actin from Sigma-Aldrich (St. Louis, MO). Human GDNF enzyme-linked immunosorbent assay kit (ELISA) was purchased from Promega (Madison, WI). Cell culture plastic ware was purchased from Corning (Lowell, MA), cell culture media and its additives from Gibco-Invitrogen. General laboratory reagents were purchased from Sigma-Aldrich unless specified in the text.

2.2. Cell lines. Baby hamster kidney (BHK-21) and rat adrenal PC12 cells were purchased from American Type Culture Collection (ATCC). Human immortalized dermal fibroblasts stably transduced with a lentivirus vector expressing hGDNF (MDX-12-GDNF) were kindly provided by professor Patrick Aebischer (Brain Mind Institute, Lausanne, Switzerland). BHK-21 cells were cultured in BHK-21 Glasgow MEM supplemented with 5% FCS, 10% tryptose phosphate broth, 2 mM glutamine, 20 mM HEPES (Complete BHK medium). MDX-12 cells were cultured in Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with 10% FCS and rat PC12 cells were cultured on collagen-coated plates (5 μg/cm^2) in D-MEM supplemented with 5% horse heat-inactivated serum, 10% FCS. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were added to all cell culture media.

2.3. Construction of the SFV-hGDNF vector. The full-length cDNA sequence of hGDNF was amplified by RT-PCR from the human astrocyte cell line SVGp12(Moretto et al., 1996) by using the following primers:

5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTTAAGATGAAGTTATGGGATGTCG-3′, and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTCAGATACATCCACACC

TTTTAGCG-3´, in which the underlined sequences hybridize with the 5´ and 3´ ends of hGDNF gene, respectively. The PCR fragment obtained in this way was cloned into plasmid pDONR201 (Invitrogen) following the manufacturer's instructions, generating pDONR201-GDNF. For the preparation of SFV-hGDNF expression vector, the hGDNF gene was amplified by PCR from plasmid pDONR201-GDNF by using the following primers: 5´-CGTAGTACGTA**cccggg**AAGTTATGGGATGTCGTGGC-3´ in which the underlined sequence hybridizes with the 5´ end of the hGDNF gene and 5´-CGTAGTACGTA**cccggg***TCA*GATACATCCACACCTTTTAGC-3´ in which the underlined sequence is complementary to the 3´ end of the hGDNF gene (stop codon in italics). A fragment of 0.67 kb was obtained, digested with Xma I (sites indicated in bold) and cloned into the unique Xma I site of pSFV-b12A (Rodriguez-Madoz et al., 2005), generating plasmid pSFV-hGDNF. The hGDNF gene was cloned downstream of the SFV subgenomic promoter fused in frame with the sequence coding for the first 34 amino acids of the SFV capsid (SFV minimal translation enhancer) and the 2A autoprotease of foot and mouth disease virus (FMDV).

2.4. hGDNF expression by BHK cells transfected with the SFV-hGDNF vector and shut-off analysis. Plasmid pSFV-hGDNF was linearized with SpeI and used as template for RNA synthesis using SP6 polymerase as previously described (Liljestrom and Garoff, 1994). Briefly, 1.5 μg of linearized pSFV-hGDNF DNA was incubated for 1 h at 37ºC in SP6 buffer supplemented with 1 mM m7G(5´)ppp(5´)G (New England Biolabs, Ipswich, MA), 10 mM DTT, 1 mM rNTP mix, 50 units of RNAse inhibitor (Promega), and 30 units of SP6 RNA polymerase (Amersham Pharmacia, UK) in a final volume of 50 μl, yielding 50 μg of RNA. About 25 μg of *in vitro* synthesized RNA were mixed with 5 x 10^6 BHK cells and electroporated in a 0.4 cm cuvette by giving two consecutive pulses at 850 V and 25 μF in a Bio-Rad electroporator (Hercules, CA). These electroporation conditions are routinely used

with the SFV system and allow transfection of > 95% cells. After electroporation, cells were diluted with 15 ml of BHK-complete medium, seeded on 6-well plates (1.5 ml/dish) and incubated at 37ºC or 33ºC with 5% CO_2. After 4-12 h (shut-off period) medium was removed, cells were washed three times with 2 ml of PBS, and 1 ml of BHK complete medium without FBS was added to each well. 24-48 h later (post-shut-off period) supernatants were collected and analyzed by Western blot with antibodies specific for hGDNF, IGFBP-4 or IGFBP-5. Lysates were also collected and analyzed by Western blot by using an antibody specific for β-actin. For protein identification 10^7 BHK cells were electroporated with 50 μg of SFV-hGDNF RNA or mock-electroporated, incubated at 33ºC during 6 h, washed three times with PBS and incubated for 24 h at 33ºC with 10 ml of BHK complete medium without FBS. Supernatants were collected and precipitated with trichloroacetic acid (TCA). Pellets were resuspended in $NaHCO_3$ at pH 8.2, digested with trypsin and analyzed by coupled liquid chromatography and tandem mass spectrometry (LC-MS/MS).

2.5. Virus production and infection. Packaging of SFV-hGDNF RNA into viral particles (vp) was performed by co-electroporation of BHK-21 cells with the vector RNA and both helper-S2 and helper-C-S219A SFV RNAs, providing the viral structural proteins in *trans*, as described (Smerdou and Liljestrom, 1999). SFV particles were purified by ultracentrifugation as described previously (Fleeton et al., 1999). The titer of recombinant virus was determined by indirect immunofluorescence of infected BHK-21 cells, using a rabbit polyclonal antiserum specific for SFV replicase as primary antibody (Casales et al., 2008). For analysis of hGDNF expression, 6-well plates containing BHK-21 cell monolayers were washed with PBS before infection. SFV-hGDNF vp were diluted in infection medium (MEM with 0.2% BSA, 2 mM glutamine and 20 mM HEPES) and added to cells. Virus adsorption was performed at 37ºC for 1 h. Cells were incubated with complete BHK-21 medium for 6 h,

washed three times with PBS and incubated with FBS-free medium. Supernatants and lysates were collected 24 h later and analyzed by Western.

2.6. Western blot analysis. SDS-PAGE was performed on NuPAGE 12% Bis-Tris Gel polyacrylamide gels (Invitrogen) under reducing conditions. Proteins were transferred onto nitrocellulose membranes and the membrane was incubated with blocking solution containing 5% non-fat dry milk in TBST (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 h, followed by incubation with anti-GDNF, anti-IGFBP-4, anti-IGFBP-5 or anti-actin antiserum diluted 1:2000 in blocking buffer for 16 h at 4ºC. After washing with TBST, a HRP-conjugated donkey anti-rabbit secondary antibody (GE Healthcare-Amersham, UK) diluted in blocking solution (1:2000) was added for 1 h. Detection was achieved with LumiLight Plus Reagent (Roche, Switzerland). Unless specified, incubations were performed at room temperature. Band densities were estimated using the software ImageJ (NIH).

2.7. Analysis of hGDNF glycosylation. For glycosylation analysis supernatants from cells electroporated with SFV-hGDNF RNA were treated with different glycosidase mixes using a glycoprotein deglycosylation kit (Calbiochem, San Diego, CA) according to the manufacture´s instructions. Briefly, samples were incubated with glycosidases during 24 h at 37ºC in the presence of 0.75% Triton X-100. After treatment samples were analyzed by Western blot as described above.

2.8. Production and purification of hGDNF. A total of 5 x 10^8 BHK cells were electroporated with 2.5 mg of SFV-hGDNF RNA synthesized *in vitro* as described previously (100 electroporations of 5 x 10^6 BHK cells with 25 μg of RNA each). Electroporated cells were pooled, resuspended in BHK-complete medium, and distributed in 50 flasks of 75 cm^2. The medium was removed after 8 h of incubation at 33ºC with 5% CO_2. Cells were washed twice with 10 ml of PBS (each flask), and BHK-complete medium without FBS was added. The supernatant from all flasks (570 ml) was collected 24 h later and centrifuged at 1000 x g

for 5 min to remove cell debris. The supernatant was adjusted to pH 8.2 with NaOH and filtered through a 0.22 μm filter unit. A SP Sepharose^{TM} Fast Flow resin (GE Healthcare) was packed into a disposable column (Bio-Rad), which was washed with 1.5 M NaCl in phosphate buffer (PB, 10 mM phosphate pH 8.2) and equilibrated with 150 mM NaCl in PB. The supernatant was passed through the column and the resin was washed with 10 column volumes (CV) of 150 mM NaCl in PB. Finally, bound protein was eluted in a single step with 2 ml (10 CV) of 0.5 M NaCl in PB. An additional elution with 10 CV of 1 M NaCl in PB was performed to ensure that all bound GDNF had been eluted from the resin. The presence of the protein was confirmed by Western blot and the purity of the sample was assessed by SDS-PAGE followed by Coomassie blue staining.

2.9. *In vitro* bioactivity assay. The bioactivity of purified hGDNF was assessed using the PC12 cell line as described previously (Garbayo et al., 2007). Briefly, PC12 cells were plated onto 12-well culture collagen-coated plates at a low density (2 x 10^3 cells/cm^2) in 1 ml of culture media. The culture medium was supplemented 24 h later with 50 ng of purified hGDNF or unglycosylated commercial hGDNF used as control. After 8 days in culture, neurite outgrowth was visualized under phase contrast illumination with a Leica DM IRB inverted microscope (Germany) connected to a Hamamatsu ORCA-ER digital camera (Japan).

3. RESULTS

3.1. hGDNF expression in BHK cells using a SFV vector

The SFV vector expressing hGDNF (SFV-hGDNF) was constructed with the hGDNF gene placed downstream the viral subgenomic promoter fused in frame with the minimal capsid translation enhancer (Fig. 1A), which codes for the first 34 amino acids of the SFV capsid and increases protein expression by 8-fold (Sjoberg et al., 1994). In order to remove the capsid enhancer, the 2A autoprotease of FMDV was used as a linker (Ryan and Drew, 1994; Smerdou and Liljestrom, 1999).

RNA was synthesized *in vitro* from pSFV-hGDNF and electroporated into BHK cells. The supernatant from transfected cells was collected 24 h later and hGDNF expression was analyzed by Western blot and ELISA. hGDNF was readily detected by Western blot in the supernatant of SFV-hGDNF transfected cells, showing a complex pattern with several specific and intense bands, most likely corresponding to different glycosylation products (Fig. 1B) (Ansorena et al., 2010; Garbayo et al., 2007). hGDNF obtained from the supernatant of human immortalized dermal fibroblasts transduced with a lentiviral vector expressing hGDNF (MDX12-GDNF) was used as positive control (Ansorena et al., 2010). Cells electroporated with RNA from a SFV vector coding for puromycin N-acetyl-transferase (SFV-pac) were used as a negative control, demonstrating that BHK cells by themselves did not express GDNF. ELISA quantification showed that BHK cells transfected with the SFV-hGDNF RNA expressed 0.6-2 μg/ml of hGDNF. This level of expression was at least twofold higher than the one obtained with the lentiviral-based system (0.3 μg/ml).

To check if the different hGDNF bands detected by Western blot were due to glycosylation, hGDNF expressed from SFV-electroporated cells was treated with N- and O-glycosidases. Removal of either N- and O-linked oligosaccharides increased the apparent electrophoretic mobility in 12% SDS-PAGE, being the effect more pronounced when both types of residues

were eliminated simultaneously (Fig. 1C). These results indicate that hGDNF expressed from SFV-transfected cells is highly glycosylated. An important feature of the SFV system is the strong shut-off of host-cell proteins synthesis which takes place in mammalian cells at 4-8 h after transfection (Strauss and Strauss, 1994). We reasoned that the shut-off could constitute an important advantage for the production of hGDNF, since SFV-hGDNF transfected cells will only secrete this protein to the medium. In order to determine the precise time for a complete shut-off, BHK cells were electroporated with SFV-hGDNF RNA or without RNA (mock) and the medium was changed to serum-free medium at 4, 6, or 8 h post-transfection. Cells were incubated for 24 additional h, the medium was collected and the presence of hGDNF was detected by Western blot (Fig. 2). In order to evaluate the degree of shut-off, cell supernatants were also analyzed by Western blot with antibodies specific for IGFBP-4 and -5, two proteins normally secreted by BHK cells. Neither of these proteins were detected in the supernatant of SFV-hGDNF transfected cells, indicating that a strong shut-off was established at 4 h post-transfection. As expected, both IGFBPs were easily detected in the supernatant of mock-transfected cells. This finding was specially important because IGFBPs are usually secreted by mammalian cell lines and have similar physicochemical properties as GDNF, being very hard to remove during the purification process. To further confirm the shut-off induced by the SFV-hGDNF vector on the inhibition of secretion proteins, supernatants of cells electroporated with SFV-hGDNF RNA, or mock-electroporated, were precipitated with TCA and analyzed by LC-MS/MS. As expected, human GDNF was only detected in the SFV-hGDNF sample. Additionally, IGFBP-4 and -5, as well as other secretion proteins including collagen alpha-1 and alpha-2, type IV collagenase, and tissue inhibitors of matrix metalloproteinase 1 and 2 were exclusively detected in mock electroporated cells.

A similar level of hGDNF expression and shut-off induction was observed in BHK cells infected with SFV-hGDNF viral particles (vp). For that purpose SFV-hGDNF was previously

packaged into vp as described in Materials and Methods. BHK cells were infected with different amounts of vp and the medium was changed by serum-free medium at 6 h post-infection. After 24 h, the presence of hGDNF and IGFBP-5 in the supernatants was analyzed by Western blot as described above (Fig. 2B). When a multiplicity of infection (MOI) ≥10 was used, hGDNF expression was comparable to that of electroporated cells, with no IGFBP-5 being detected, indicating that a strong shut-off had been induced. These results showed that hGDNF expression could be achieved by either electroporation or infection of cells with SFV vectors.

3.2. Optimization of hGDNF expression

In order to determine the best conditions for hGDNF production, BHK cells were electroporated with SFV-hGDNF and incubated under different conditions which included: (i) incubation time after electroporation or "shut-off" period, (ii) incubation time after changing the initial culture medium for serum-free medium, or "postshut-off" period, and (iii) incubation temperature. The presence of hGDNF was analyzed directly by Western blot from the conditioned medium. Secreted proteins were precipitated with TCA in order to detect IGFBP-4 expression with higher sensitivity. The ratio between the hGDNF and IGFBP-4 band intensity was determined for each condition and used as a measurement of the shut-off degree (Table 1). The highest hGDNF expression was achieved when cells were incubated at 33ºC. A shut-off period of at least 8 h was necessary to completely inhibit IGFBP-4 expression, regardless of the incubation temperature. Although the transfection efficacy was very high (above 90%), prolongation of the shut-off over 24 h resulted in some expression of IGFBP-4 probably due to the growth of untransfected cells. Therefore, the conditions selected for further hGDNF expression were 33ºC and 8 h of shut-off followed by 24 h of postshut-off incubation (in bold in Table 1).

3.3. hGDNF purification

In order to purify hGDNF, a total of 5 x 10^8 BHK cells were electroporated with SFV-hGDNF RNA and incubated at 33ºC. After 8 h the medium was removed and substituted by serum-free medium and cell supernatant was collected 24 h later. hGDNF concentration in this medium was 1.3 μg/ml as measured by ELISA. Since hGDNF has a theoretical pI of 9.26 the pH was adjusted to 8.2. Positively charged hGDNF was purified by passing the supernatant through a cation exchanger and eluting the recombinant protein with 0.5 M NaCl. Analysis of the eluate by SDS-PAGE followed by Coomasie Blue staining showed the presence of a double band with a molecular weight similar to hGDNF (Fig. 3A). Western blot analysis (Fig. 3B) confirmed that (i) this double band corresponded to hGDNF and that (ii) purified hGDNF exhibits a complex pattern of bands which likely corresponds to different degrees of glycosylation (Garbayo et al., 2007). Quantification of purified protein by ELISA showed that a total amount of 0.445 mg of hGDNF had been obtained from 0.57 L of medium, with a 60% recovery yield.

3.4. Activity of purified hGDNF

The ability of purified hGDNF to induce neurite outgrowth on PC12 cells was used as an indicator of bioactivity (Fig. 4). Neurite outgrowth was clearly observed in PC12 cells incubated with 50 ng/ml of purified hGDNF. These results indicate that the recombinant neurotrophic factor obtained with the SFV expression system is biologically active. The degree of activity was similar to that of commercial unglycosylated hGDNF expressed in bacteria. In the absence of hGDNF, PC12 cells displayed the typical undifferentiated morphology.

4. DISCUSSION

The present work describes a simple and efficient procedure for the expression and purification of hGDNF. One of the novelties of the study is the use of an alphavirus expression system to produce high levels of hGDNF. The absence of host-cell proteins in the supernatant of mammalian cells was achieved taking advantage of the strong shut-off of host-cell protein synthesis induced by alphavirus vector replication in transfected cells. A second important achievement was the production of highly glycosylated hGDNF, which is more similar to the endogenous hGDNF than the protein produced in *E. coli*.

In spite of good expression levels, purification of GDNF from the culture medium of mammalian cell lines is a long process that requires several chromatographic steps (Ansorena et al., 2010; Garbayo et al., 2007). The main handicap of the process is the high basal expression of endogenous proteins belonging to the IGFBP family which seem to be secreted constitutively by mammalian cells. The physicochemical properties (pI and molecular weight) of these proteins are similar as those of GDNF. Removal of these contaminants during the purification process results in a tremendous decrease in the GDNF purification yield, making this technology inefficient to obtain high amounts of GDNF for clinical purposes.

It has been shown that alphavirus vectors can express high levels of cytoplasmic, transmembrane, and secretion proteins (Liljestrom and Garoff, 1991). However, to our knowledge, large-scale production and purification of proteins using alphaviruses has only been reported for cytoplasmic and transmembrane proteins (Blasey et al., 1997; Lundstrom, 2003; Lundstrom and Ehrengruber, 2003). Production of secretion proteins could benefit from the fact that a few hours after transfection or infection with an alphavirus vector, a strong shut-off of host-cell protein synthesis is induced in such a way that cells mainly produce the heterologous protein expressed by the vector. This feature of alphavirus replication could

represent a great advantage for expression of secreted proteins, since the substitution of cell medium by new serum-free medium once the shut-off has been fully established, would lead to the accumulation of the recombinant protein expressed by the vector in the supernatant. However, to reach a good efficacy with this process, it is necessary to deliver the alphavirus vector to most cells in the culture, since the shut-off will not be effective in untransfected or uninfected cells. Although shut-off induction mechanisms are not completely understood it seems that vector RNA replication is recognized in infected cells as a danger signal, inducing activation of protein kinase R, which will phosphorylate eukaryotic initiation factor 2 alpha (eIF2α), inhibiting protein translation (Ventoso et al., 2006). To be able to express their structural proteins under such circumstances some alphavirus, like SFV, contain a cis-acting sequence at the beginning of the structural ORF that allow eIF2α–independent translation. This sequence, known as translation enhancer, has also been used in our vector to induce high hGDNF expression. As expected, BHK cells electroporated or infected with the SFV-hGDNF vector did not significantly produced host-cell secreted proteins, like IGFBP-4 and IGFBP-5, collagen or matrix metalloproteinase inhibitors (Figure 2 and LC-MS/MS analysis). Therefore, high levels of nearly pure hGDNF were accumulated in the supernatant. The optimization study showed that the shut-off was complete 8 h after electroporation. After serum-free medium addition, an incubation time of 24 h was found to be optimal for hGDNF production. Longer incubation times resulted in higher levels of hGDNF but also in the reappearance of contaminant proteins in the culture medium, probably due to cell lysis induced by the SFV vector, as well as to the growth of a small amount of untransfected cells. A higher hGDNF expression was achieved by incubating SFV transfected cells at 33ºC compared to 37ºC as it has been reported by Schlaeger and Lundstrom (Schlaeger and Lundstrom, 1998). Purification of hGDNF was very simple, obtaining pure glycosylated hGDNF in a single chromatographic step. Using this system, 0.78 mg of purified hGDNF

were obtained per liter of supernatant, with a 60% recovery yield. As it has been shown, hGDNF expression in the absence of contaminating proteins can be achieved by either electroporation or infection of cells with the SFV-hGDNF vector. In the latter case vector RNA is previously packaged into viral particles by co-transfecting cells with two helper RNAs providing the structural proteins in *trans* (Smerdou and Liljestrom, 1999). This process led to the generation of 1.8 x 10^{10} vp of SFV-hGDNF from a single electroporation of 10^7 cells (data not shown). This amount of vp could be used to infect 3.6 x 10^9 cells, using a MOI of 5 to ensure that all cells are infected, leading to a potential production of approximately 3.2 mg of hGDNF. Expressing hGDNF from infected cells would have several advantages with respect to electroporation: i) large amounts of SFV vp could be generated allowing hGDNF production to be scaled up, ii) only a small amount of RNA is needed to produce a large stock of SFV-hGDNF vp, consecuently the overall cost would be reduced, iii) it is possible to avoid the growth of non-transfected cells and the expression of their endogenous proteins by achieving a 100% infection using the appropriate number of SFV vp. Although in this study we have used adherent cells, both vector electroporation and infection could be performed with cells growing in suspension for a better scaling up.

Glycosylation, a post-translational modification affecting secreted and transmembrane proteins, plays a role on protein activity, half-life or diffusion through the extracellular matrix. For therapeutic proteins this modification could affect immunogenicity, solubility, stability or pharmacodistribution (Wadhwa et al., 1996) GDNF contains two N-linked glycosylation sites and it has been described that N-linked glycans account for up to approximately 25–35% of the molecular mass of the mature protein (Lin et al., 1994). We have observed that recombinant hGDNF also contains O-linked glycans. Complete elimination of both N- and O-linked glycans results in a band of approximately 15 kDa, which corresponds to the size of the mature protein. Although glycosylation of hGDNF does not seem to increase its biological

activity (Fig. 4), this post-modification could be very useful for the treatment of human diseases preventing the generation of neutralizing antibodies (Arnau et al., 2006).

5. CONCLUSION

Although some recombinant proteins are already being used as therapeutic molecules, there is a requirement for the development of more effective purification processes. In a competitive pharmaceutical market where production costs are high, the development of more economical production and purification procedures would add a significant advantage and a trend to follow. This work describes a novel and efficient procedure for the expression and purification of hGDNF based on a SFV vector. hGDNF is a secreted and highly glycosylated protein with neuroprotective and regenerating properties that is currently under investigation in clinical trials for the treatment of neurodegenerative disorders. Interestingly, the procedure described in this study could be extended to other secreted proteins and could be scaled up for industrial purposes.

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FIGURE LEGENDS

Figure 1. hGDNF expression using a SFV vector. (A) The vector contains the SFV replicase followed by a subgenomic promoter (sg Pr). The ORF coding for hGDNF is placed downstream of the sg Pr fused to the minimal SFV capsid translation enhancer (Enh b1) and the FMDV 2A autoprotease (2A). Enh b1 codes for the 34 first amino acids of SFV capsid and 2A codes for the 17 amino acids indicated in the figure (the arrow below the figure indicates the protease cleaving site). (B) BHK cells were electroporated in duplicate with hGDNF RNA and the presence of hGDNF was analyzed in supernatants 24 h later by Western blot (lanes 1 and 2). Cells electroporated with RNA from a SFV vector coding for puromycin N-acetyl-transferase were used as negative control (SFV-pac). Supernatant from MDX-12 cells expressing hGDNF (lenti hGDNF) was used as positive control. (C) Deglycosylation analysis of hGDNF. SDS-PAGE and Western blot analysis of supernatant

from cells electroporated with SFV-hGDNF after treatment with N or O-endoglycosidases. w/o, treatment without glycosidases; N, treatment with N-glycosidase F; O, treatment with a mix of O-glycosidases (Endo-α-N-acetylgalactosaminidase, α2-3.6.8.9-neuraminidase, β1.4-galactosidase and β-N-acetylglucosaminidase); N+O, treatment with both N- and O-glycosydases.

Figure 2. Shut-off analysis in SFV-hGDNF transfected or infected cells. (A) BHK cells were electroporated with SFV-hGDNF RNA in duplicate (Electrp 1 and 2) or without RNA (mock). (B) BHK cells were infected with SFV-hGDNF vp at the indicated MOIs, using cells electroporated with SFV-hGDNF RNA as positive control (Electrp). Medium was changed to serum-free medium at the times indicated in A, or at 6 h in B, collected 24 h later and analyzed by Western blot with antibodies specific for hGDNF, IGFBP-5 and IGFBP-4. Cell lysates were also collected and analyzed by Western blot with an antibody against β-actin, as loading control.

Figure 3. hGDNF purification. 5 x 10^8 BHK cells were electroporated with SFV-hGDNF RNA, incubated at 33ºC and after 8 h the medium was replaced by serum-free medium. Cells were incubated at 33ºC for 24 h, the supernatant was collected, adjusted to pH 8.2 and passed through a SP-Shepharose fast flow resin. Bound hGDNF was eluted in a single step with 0.5 M NaCl, the eluate was analyzed by Coomasie Blue staining (A) and Western blot with an antiserum specific for hGDNF (B). L: Supernatant loaded onto the column: FT: Flow through; W: column wash; E1: eluate with 0.5 M NaCl; E2: eluate with 1 M NaCl.

Figure 4. Analysis of hGDNF activity. PC12 cells were plated on collagen at low density (2 x 10^3 cells/cm^2) and incubated for 8 days with 50 ng/ml of hGDNF purified from the supernatant of BHK cells transfected with SFV-hGDNF vector or with unglycosylated hGDNF produced in *E. coli*. Phase contrast images were taken on day 8.