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Production of highly pure human glycosylated GDNF in a mammalian cell line

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ABSTRACT

The administration of glial cell line-derived neurotrophic factor (GDNF) has emerged as a promising strategy for the treatment of several diseases of the nervous system as Parkinson's disease, amyotrophic lateral sclerosis, spinal cord injury and nerve regeneration as well as ocular diseases and drug addictions. A procedure for the purification of human recombinant glycosylated GDNF using a mammalian expression system as the source of the protein is discussed in the present paper. The neurotrophic factor was purified using cation exchange chromatography and gel filtration. A human cell line was chosen as the source of therapeutic protein, since a recombinant protein with a structure and glycosylation pattern equivalent to the native form is desirable for its prospective therapeutic utilization. The activity of the highly pure protein obtained was confirmed with a cell-based bioassay. The purified protein is suitable for its *in vivo* evaluation in animals and for possible subsequent clinical application.

1. Introduction

Therapeutic proteins constitute a fast-growing category in the arsenal of drugs, and represent one of the fastest sales growth areas within pharmaceuticals. Due to their high specificity and activity, new therapies superior to conventional drugs could be designed, and diseases that are untouched by small-molecule drugs could be treated.

Biologically active proteins with potential for therapeutic delivery include hormones, enzymes, antibodies, antigens and growth factors. Among them, neurotrophic factors are naturally occurring proteins implicated in the differentiation and survival of the nervous system during development, and are also essential for the maintenance, survival and repair of neuronal function in adulthood. Neurotrophic factors have therefore been advocated as ideally suited for the treatment of neurodegenerative diseases of the central nervous system, neuropathies and peripheral nerve injury (Terenghi, 1999).

One of those neurotrophic factors, the glial cell line-derived neurotrophic factor (GDNF), is a member of the transforming growth factor-β superfamily that has been shown to be neuroprotective, it has enhanced nerve regeneration and it has presented potent neuronal survival-promoting and trophic effects on various neuronal populations including dopaminergic (Lin et al., 1993; Hoffer et al., 1994) noradrenergic (Arenas et al., 1995), cortical (Wang et al., 1997; Giehl et al., 1998), retinal ganglion (Klocker et al., 1997; Yan et al., 1999), sensory (Molliver et al., 1997; Bennett et al., 1998; Blesch and Tuszynski, 2003) and motor neurons (Henderson et al., 1994; Oppenheim et al., 1995; Hottinger et al., 2000). All these reasons have prompted the use of this protein as a therapeutic agent in several neural diseases. At a preclinical level, the administration of GDNF has been tested with promising results in animal models of amyotrophic lateral sclerosis (Mohajeri et al., 1999; Ciriza et al., 2008; Suzuki et al., 2008), spinal cord injury (Cheng et al., 2002; Sharma, 2006; Guzen et al., 2009) and for the treatment of peripheral nerve injuries (Piquilloud et al., 2007), including pure motor nerves such as the facial nerve (Barras et al., 2002; Shi et al., 2008), and mixed sensory and motor nerves such as the sciatic nerve (Chen et al., 2001; Wood et al., 2009). It has also been evaluated in animal models of ocular diseases such as glaucoma (Ward et al., 2007; Jiang et al., 2007), retinal ischemic injuries (Wu et al., 2004), the prevention of the retinal ganglion degeneration induced after optic nerve transection (Yan et al., 1999; Schmeer et al., 2002) and other models of retinal degeneration (Buch et al., 2006). Moreover, as reviewed in Carnicella and Ron (2009), GDNF plays a unique role in negatively regulating the actions of drugs of abuse, including psychostimulants, morphine and alcohol, and the GDNF pathway has also been proposed as a potential target for the treatment of drug addiction.

In a further step, on a clinical level, GDNF has been tested in several clinical trials for Parkinson's disease (Gill et al., 2003; Patel et al., 2005; Lang et al., 2006). The recombinant GDNF used in all these clinical trials was produced in *Escherichia coli*, and some patients enrolled in the phase II clinical trial developed anti-GDNF

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antibodies, against the exogenous protein, owing to lack of glycosylation of the protein (Slevin et al., 2007). Like 60% of therapeutic proteins (Gerngross, 2004), GDNF is a glycosylated protein, and consequently the best choice for its potential therapeutic utilization in terms of efficacy and safety would be the administration of a protein with a structure and glycosylation pattern equivalent to the native form.

Nowadays, recombinant human GDNF is available from *E. coli*, and some authors have described a procedure for the expression and purification of human GDNF from *Trichoplusia ni* (Tn-5B1-4) insect cells infected with baculovirus (Chen et al., 2000c; Trupp et al., 1995), and the expression of human GDNF in *Pichia pastoris* culture medium (Chen et al., 2000b), but prokaryotic hosts do not glycosylate proteins and lower eukaryotic expression systems, such as insect cells or yeasts, are typically unable to provide a mammalian glycosylation pattern (Gerngross, 2004). Thus, the majority of therapeutic proteins are produced in either mammalian cell-culture systems or in *E. coli* (Chu and Robinson, 2001; Swartz, 2001; Jana and Deb, 2005), but it must be borne in mind that over half of the biotechnology products approved by the FDA from January 1996 to November 2000 (21 out of 33) were manufactured using mammalian cell systems, as they represent the best method for obtaining the greatest degree of product fidelity (Chu and Robinson, 2001).

The aim of this study was to develop a procedure for the production and purification of human bioactive glycosylated GDNF using a mammalian cell expression system.

2. Materials and methods

2.1. Materials

Human recombinant GDNF was obtained from Invitrogen (Carisbad, CA, USA). Polyclonal antibody anti-GDNF was from Santa Cruz biotechnology (Heidelberg, Germany). GDNF enzyme linked immunosorbant assay kit (ELISA) was purchased from Promega (Madison, USA). The adrenal rat PC12 cell line was purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). MDX-12 hGDNF secreting cell line, were provided by Professor Patrick Aebischer (EPFL, Lausanne, Switzerland). Cell culture plasticware was purchased from Corning (Amsterdam, The Netherlands) and cell culture media and its additives from Gibco-Invitrogen (Paisley, Scotland, UK). Silver Stain Plus was obtained from BioRad (California, USA). General laboratory reagents were purchased from Sigma–Aldrich (Barcelona, Spain) unless specified in the text.

2.2. Cell culture

MDX-12 were cultured in Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with 10% fetal bovine heat-inactivated serum (FBS), 100 units/ml of penicillin and 100 μg/ml of streptomycin. Rat PC12 cells were cultured on collagen coated plates (5 $\mu g/cm^2$) in D-MEM supplemented with 5% horse heat-inactivated serum, 10% FBS, 100 units/ml of penicillin and 100 μg/ml of streptomycin.

2.3. Production and purification of hGDNF

Human immortalized dermal fibroblasts transduced with a pCCL-WPS-hGDNF lentiviral vector (MDX-12), were kindly provided by professor Patrick Aebischer (Sajadi et al., 2006) (EPFL, Lausanne, Switzerland). When the cells reached 80% confluence, they were washed with PBS and changed to fresh serum-free medium. After 48 h, conditioned medium was harvested, filtered though a 0.22 μm filter unit and adjusted to pH 8.2 with NaOH. Protein expression levels were analyzed by SDS-PAGE followed by Western Blot (WB) under reducing conditions (50 mM dithiothreitol and 5% β-mercaptoethanol).

The secreted protein was purified by two chromatography steps as follows:

1. Cation exchange chromatography
   A XK 16/20 column (GE Healthcare) was packed with SP Sepharose™ Fast Flow resin (GE Healthcare Uppsala, Sweden), charged with 1.5 M NaCl in equilibration buffer (10 mM phosphate, 5 mM EDTA), and equilibrated with 150 mM NaCl in equilibration buffer, pH 8.2. The conditioned medium was passed through the column and bounded proteins were eluted using a linear salt gradient from 150 mM NaCl to 1 M NaCl in equilibration buffer. All the procedure was performed at 4 °C to avoid protein degradation. hGDNF containing fractions were identified by WB. The purity of the protein was assessed by Coomassie blue (CB) staining. hGDNF enriched fractions were passed again through the same column under the same conditions.
2. Gel filtration chromatography
   hGDNF fractions were pooled and desalted to 50 mM NaCl, 10 mM phosphate buffer pH 8.2 using a HiPrep™ 26/10 Desalting (GE Healthcare) prior to concentration by lyophilization. Finally, the sample was resuspended in ultrapure water and then applied to a Superdex™ 200 HR 10/30 column (GE Healthcare) using an ÁKTA purifier 10 (GE Healthcare) at a flow rate of 0.5 ml/min. The column was previously calibrated using the Molecular Weight market kit Sigma–Aldrich (Steinheim, Germany) including aprotinin, cytochrome *c*, carbonic anhydrase, albumin and blue dextran. Fractions of 0.5 ml were collected and analyzed by WB and CB to determine hGDNF elution and purity.

2.4. Identification of the proteins by mass spectrometry

An excised gel slice from the SDS-PAGE gel stained with CB was destained with 50 mM ammonium bicarbonate/50% (vol/vol) acetonitrile. Then, proteins were reduced with 10 mM DTT in 100 mM ammonium bicarbonate and alkylated with 55 mM iodoacetamide in the same buffer. In-gel protein digestion was performed with 6 ng/μl trypsin in 50 mM ammonium bicarbonate for 5 h at 37 °C. Peptide mass fingerprinting was obtained from tryptic digests by LC-ESI–MS/MS analysis. Microcapillary reversed phase LC was performed with a CapLC™ (Waters) capillary system. Reversed phase separation of tryptic digests was performed with an Atlantis, C_{18}, 3 μm, 75 μm × 10 cm Nano Ease™ fused silica capillary column (Waters) equilibrated in 5% acetonitrile, 0.2% formic acid. After injection of 6 μl of sample, the column was washed for 5 min with the same buffer and the peptides were eluted using a linear gradient of 5–50% acetonitrile in 30 min at a constant flow rate of 0.2 μl/min. The column was coupled online to a Q-TOF Micro (Waters) using a PicoTip nanospray ionization source (Waters). The heated capillary temperature was 80 °C and the spray voltage was 1.8–2.2 kV. MS/MS data were collected in an automated data-dependent mode. The three most intense ions in each survey scan were sequentially fragmented by collision induced dissociation (CID) using an isolation width of 2.5 and a relative collision energy of 35%. Data processing was performed with Mascot Search (for the GDNF) and Proteinlynx Global Server 2.1 (Waters) (for the IGFBP7) against Uniprot knowledegebase Release consisting of UniprotKB/Swiss-Prot Release and UniprotKB/TrEMBL Release. The search was enzymatically constrained for trypsin and allowed for one missed cleavage site. Further search parameters were as follows: no restriction on molecular weight and isoelectric point; fixed modification, carbamidomethylation of cysteine; variable modification, oxidation of methionine.

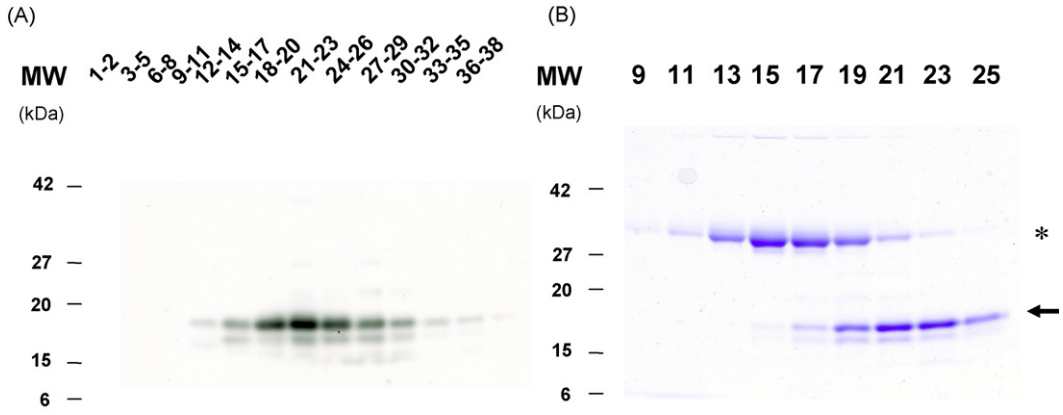

Fig. 1. GDNF analysis after cation exchange chromatography. Bound GDNF was eluted with a salt gradient from 0.15 to 1 M NaCl. Fractions (2.5 ml) were analyzed by SDS-PAGE under reducing conditions followed by WB (A) and CB staining (B). Black arrow shows GDNF. Asterisk shows IGFBP7.

2.5. N-Glycanase digestion

Two micrograms of purified GDNF were resuspended in 30 μl of PBS and denatured in 20 mM phosphate pH 7.5, 0.1% SDS and 50 mM β-mercaptoethanol by heating at 100 °C for 5 min. Samples were then incubated on ice for 5 min, and NP-40 (0.75%) was added. Afterwards, 0.0025 U of N-glycosidase F, Prozyme (San Leandro, CA, USA) was added and samples were incubated at 37 °C for 6 h. As a control, two micrograms of the same aliquot of purified GDNF were incubated in the absence of N-glycosidase. Results of the reactions were analyzed by WB.

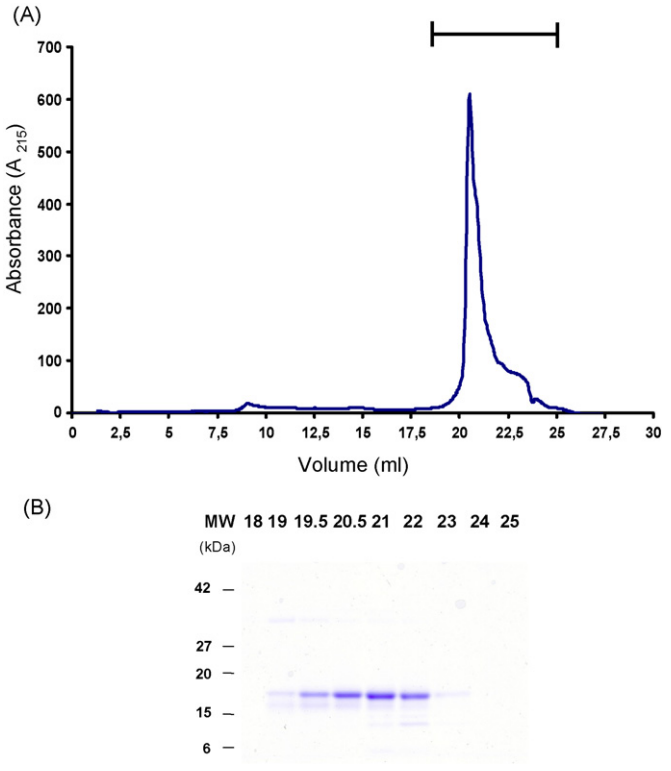

Fig. 2. GDNF analysis after size exclusion chromatography. (A) Protein profile (absorbance at 215 nm) over volume (ml) from the gel filtration column chromatography showing a major peak that corresponds to the fractions containing GDNF. Those fractions were analyzed by SDS-PAGE followed by CB staining of the gel, in order to determine the final purity of the samples (B). The bar indicates the fractions that were analyzed by CB.

2.6. Western blot analysis

SDS-PAGE was performed on NuPAGE 12% Bis-Tris Gel polyacrylamide gels (Invitrogen, Carlsbad, USA) under reducing conditions. Proteins were transferred onto nitrocellulose membranes. The membrane was incubated with the blocking solution: 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 the incubation with anti-GDNF antiserum 1:2000 in blocking buffer for 16 h at 4 °C. After washing with TBST, the secondary antibody donkey anti-rabbit HRP 1:2000 (GE Healthcare-Amersham) was added in blocking solution for 1 h. Detection was achieved with Lumi Light Plus Reagent (Roche). Unless specified, incubations were performed at room temperature.

2.7. In vitro bioactivity assay

The bioactivity of the purified GDNF was assessed using the PC12 cell line as previously described (Garbayo et al., 2007). PC12 cells differentiate to a neural phenotype in response to neurotrophic factors such as GDNF or NGF (Garbayo et al., 2007; Cao and Schoichet, 1999). Briefly, PC12 cells were plated onto 12 well culture plate at a low density, 2×10^3 cells/cm^2 in 1 ml of culture media. The culture medium was supplemented 24 h later with 50 ng of purified GDNF, which had previously been quantified by ELISA, or 50 ng of commercial GDNF as control. After 7 days in culture, neurite outgrowth was visualized under phase contrast illumination with a Leika DM IRB inverted microscope connected to a Hamamatsu ORCA-ER digital camera.

3. Results

3.1. GDNF purification

Human GDNF was purified from the human immortalized dermal fibroblast hGDNF secreting cell line MDX-12 (Sajadi et al., 2006). Cells were incubated in medium without serum to collect secreting GDNF. The presence of the protein in the conditioned serum-free medium was established to last for up to 1 month in the culture conditions employed. These results were confirmed by SDS-PAGE developed under reducing conditions, followed by WB (data not shown). After the cation exchange chromatography, the fractions obtained were analyzed by WB to detect which ones contained the protein (Fig. 1A). Most hGDNF was obtained among fractions 17–27, which means that eluted at approximately 0.6 M NaCl (Fig. 1A). The theoretical pI = 9.26 of the protein was in agreement with the fact that GDNF bound tightly to the cation exchange resin at pH 8.2. CB staining of the gel (Fig. 1B) revealed the pres-

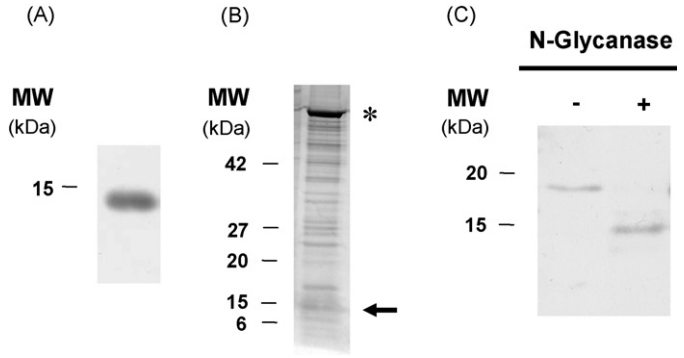

Fig. 3. Analysis of commercial (A and B) and purified human recombinant GDNF. (A) WB of the commercial hGDNF produced in *E. coli* showing the band corresponding to the size of the unglycosylated protein. (B) Image of the commercial prokaryotic GDNF. Bacterial protein was subjected to SDS-PAGE followed by silver staining under reducing conditions. Asterisk indicates BSA used to stabilize the protein and black arrow the band that correspond to the unglycosylated GDNF. (C) Treatment with N-glycanase of mammalian recombinant GDNF analyzed by SDS-PAGE and WB. In the absence of N-glycanase the main band observed has a molecular weight of 18 kDa. N-glycanase treatment results in the disappearance of the 18 kDa and the appearance of a 15 kDa band corresponding to the size of the unglycosylated protein.

ence in the sample of another protein whose peak of expression was found in fraction 16, whereas peak of expression of GDNF was in fraction 22. However, since both proteins seem to have a similar affinity for SP-Sepharose, there was a wide overlap between them. Using mass spectrometry we identified this contaminant as IGFBP7, a protein with a theoretical pI = 8.25. Fractions from 23 to 32 were pooled, desalted and lyophilized. In the next purification step, the sample was injected into the Superdex^{TM} 200 HR 10/30 chromatography column. The chromatogram shows a single peak at 20 ml postinjection, corresponding to the GDNF homodimer (Fig. 2A). The asymmetrical shape of the peak is probably due to the different glycosylation pattern of the GDNF. In order to determine the degree of purity of the purified protein, samples were analyzed by SDS-PAGE followed by CB staining of the gel (Fig. 2B). Finally, mass spectrometry studies confirmed the identification of the purified protein. The purified protein was identified as human GDNF with 7 peptides and with a score of 339 (individual ions scores >24 indicate identity or extensive homology ($p < 0.05$), mapping the 39% of the protein. All fractions containing GDNF were pooled and lyophilized.

3.2. Characterization of purified GDNF

The purity of the recombinant protein obtained was visualized by electrophoretic analysis of SDS-PAGE followed by CB. As shown in Fig. 2B, GDNF presented a unique protein band with an apparent size of 18 kDa, which was the most abundant form of the monomeric protein when analyzed by WB under reducing conditions. Commercial human bacterial GDNF was subjected also to SDS-PAGE under reducing conditions, followed by WB (Fig. 3A) and silver staining (Fig. 3B) in order to compare the purity and characteristics of the commercial GDNF. The purified protein presented a higher degree of purity compared to the commercial one, as in the purified protein it was not possible to detect traces of other protein contaminants whereas the commercial one exhibited multiple bands corresponding to non-specific proteins in the gel apart from the GDNF.

There were also differences in the size, due to the glycosylation of the purified protein. GDNF contains two potential glycosylation sites and the appearance of different molecular size bands due to N-glycosylation has been previously described (Lin et al., 1994; Trupp et al., 1995). To confirm that this was the case and that the recombinant protein presented a mammalian glycosylation pattern, purified GDNF was treated with N-glycanase (Fig. 3C). Deglycosylation reduced the apparent molecular mass to the predicted 15 kDa, the same molecular weight that presented the unglycosylated commercial protein.

3.3. Cell-based bioassay

To test the bioactivity of the protein purified, a neuronal cell-based bioassay was performed based on the ability of the protein to induce the differentiation of PC12 cells to a neural phenotype (Garbayo et al., 2007; Cao and Schoichet, 1999). Cell differentiation activity was tested after a 7-day treatment. Untreated PC12 cells presented a round shape, characteristic of undifferentiated cells (Fig. 4A). However, cells treated with 50 ng/ml of both, purified glycosylated hGDNF (Fig. 4B) or commercial unglycosylated hGDNF (Fig. 4C), differentiated to a neuron-like morphology, experiencing a marked neurite outgrowth. The differentiating activity between GDNF molecules from different origins was similar. The assay confirmed that the recombinant purified GDNF was bioactive and that the glycosylation is not required to exert its differentiating properties.

4. Discussion

Glial cell line-derived neurotrophic factor (GDNF) is widely recognized as a potent survival and regenerating factor for several neuronal populations involved in diverse human diseases. As a result, GDNF has become an important promising therapeutic protein with diverse potential medical applications. Some of them have already been introduced in clinical trials and others will probably reach the clinical phase in the next few years.

GDNF is a glycosylated, disulfide-bonded homodimer, with a molecular weight of 33–45 kDa, while the monomer has a molecular weight of 15 kDa after deglycosylation (Lin et al., 1994). There exist two N-linked glycosylation sites in mature GDNF protein (Lin

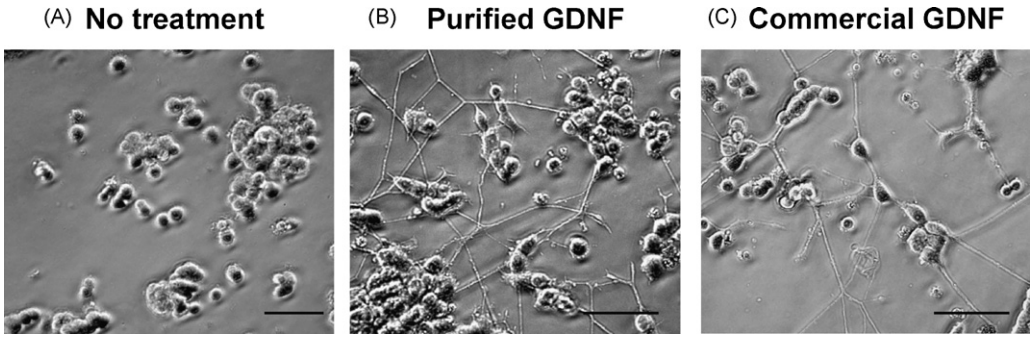

Fig. 4. Neuronal bioassay. Differentiation of PC12 cells induced by GDNF. PC12 cells were plated on collagen at a low density (2×10^3 cells/cm^2), and 24 h later cultured in the presence or absence of 50 ng/ml of purified glycosylated or commercial unglycosylated GDNF as indicated. Phase contrast images were taken on day 7. Bar length 100 μm.

et al., 1993). Although N-linked glycans account for up to approximately 25–35% of the molecular mass of GDNF, they seem not necessarily to be critical for its neurotrophic effect, since the intrastriatal administration of human non-glycosylated GDNF (obtained from *E. coli*) using mechanical osmotic pumps, has been reported to induce clinical improvement in several clinical trials, in which sprouting of dopaminergic fibers has been described (Gill et al., 2003; Patel et al., 2005; Lang et al., 2006; Slevin et al., 2005). Nevertheless, the use of a recombinant protein obtained from a human cell line with a structure equivalent to the native form is still desirable for therapeutic utilizations (Lee et al., 2008). In fact, it has been proposed that the lack of glycosylation of the protein in the clinical trials of Parkinson's disease could be the responsible for the appearance of anti-GDNF antibodies in 10% of the patients enrolled in the last phase II clinical trial (Lang et al., 2006), which was halted at least in part owing to safety concerns. There exist precedents in which the immunogenicity of biopharmaceutical proteins has been related to deglycosylation (Schellekens, 2002), including granulocyte–macrophage colony-stimulating factor, where the increased immunogenicity of the non-glycosylated bacteria or yeast-derived protein is thought to be caused by the exposure of antigenic sites which are normally protected by glycosylation on the native protein (Gribben et al., 1990), and IFN-β, wherein the higher immunogenicity of the *E. coli*-derived product compared with the mammalian-cell product has been explained by the reduced solubility of the non-glycosylated bacterial product (Karpusas et al., 1998). In this case, the carbohydrate side chains are thought to shield hydrophobic sites of mammalian IFN-β.

The exact impact of the antibodies on patients is unclear, but these antibodies not only could decrease the potency and efficacy of the therapeutics but, more importantly, could also cross-react with and block the action of the endogenous protein (Tatarewicz et al., 2007). Due to this safety factor, and other possible biochemical and pharmaceutical reasons, such as stability, solubility, ligand binding, biological half-life, pharmacodynamics and pharmacokinetics, among other properties in which the sugar component of the protein could directly affect or influence, the use of a glycosylated protein presenting a mammalian glycosylation pattern like that obtained in this study, is preferred.

Thus, although production of recombinant proteins in bacteria is rapid and economical, the use of this expression system for therapeutic proteins, especially for those that incorporate post-translational modifications, should be prevented, since these prokaryotic production systems are not able to introduce these modifications. Moreover, it has been reported that recombinant expression of GDNF in bacteria results in a product that is primarily misfolded and therefore not fully functional (Chen et al., 2000a). Some authors have developed a procedure for the expression and purification of human GDNF from *T. ni* (Tn-5B1-4) insect cells infected with baculovirus (Chen et al., 2000c; Trupp et al., 1995), and the expression of human GDNF in *P. pastoris* (Chen et al., 2000b) culture medium, but neither insect cells (that usually lack fully elaborated complex N-linked glycans (März et al., 1995; Jarvis et al., 1998) nor yeast (in which N-glycosylation is of the high-mannose type, which confers a short half-life *in vivo* and thereby compromises its efficacy (Wildt and Gerngross, 2005) are the most suitable hosts for expressing human glycoproteins, as the glycosylation in these cells is markedly different from that in humans. All these reasons argue for the expression of therapeutic recombinant proteins in eukaryotic expression hosts like the one described here, regardless of the technical difficulties that work with these systems presents.

From the technical point of view, this procedure including two cation exchange chromatographic steps and a gel filtration chromatographic step also represents a simplification compared with the procedure developed by Chen et al. (2000c) for the purification of human glycosylated GDNF produced in insect cells. In the method described by Chen, affinity chromatography and gel filtration chromatography was performed. For the affinity chromatography, a previous step of production of His-tagged GDNF in *E. coli* and purification by nickel–agarose was needed in order to immunize rabbits with the recombinant protein to generate polyclonal anti-GDNF antiserum and subsequent purification of anti-GDNF antibodies was carried out, in order to form the affinity matrix required for the chromatography.

As previously mentioned, a human cell line was used as the source of protein, and as such, the recombinant protein purified is expected to be non-immunogenic. Moreover, the purified recombinant protein is highly pure compared to the commercial protein produced in *E. coli*. The mammalian glycosylation pattern, compared to the non-glycosylated GDNF produced in bacteria and the altered glycosylation pattern produced in insect cells increases the safety of the treatment.

5. Conclusion

This is the first report describing a procedure for the production and purification of human GDNF using a human cell line as the source of the therapeutic protein. The neurotrophic factor obtained has been identified by mass spectrometry and is highly pure, bioactive, and presents a glycosylated mammalian pattern. The purified protein is suitable for its *in vivo* evaluation in diverse animal models of various diseases and for its subsequent clinical application.

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