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ORIGINAL PAPER

H. Liu · R.P. Iacono · A.A. Szalay

Detection of GDNF secretion in glial cell culture and from transformed cell implants in the brains of live animals

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Abstract Current ex vivo gene therapy for Parkinson's disease using glial cell line-derived neurotrophic factor (GDNF) is limited by the lack of a monitoring mechanism to determine the expression of GDNF once the cells or other vehicles are transferred into animal models. The purpose of this study was to test whether a *Renilla* luciferase (RUC)-GDNF fusion protein secreted by the genetically engineered glial cell line RG-1 could be measured photometrically in cerebrospinal fluid (CSF). RG-1 was constructed by permanent transformation with a plasmid DNA construct that contains a GDNF cDNA (*gdnf*) fused to a RUC cDNA (*ruc*). The fusion protein secreted by RG-1 was shown to retain both GDNF and RUC activity. The concentration of GDNF determined by enzyme-linked immunoadsorbent assay (ELISA) was correlated with the light emission detected by assaying for RUC bioluminescence in RG-1 culture medium, indicating that RUC can be used as a reporter for GDNF in vitro. The cells were then implanted into rat brain (n = 20), and the cisternal CSF was analyzed. Bioluminescence was successfully detected in the CSF samples, and was quantified over a period of 25 days, while Western blotting and ELISA failed to detect GDNF in CSF, presumably because the concentration of the RUC-GDNF fusion was too low. This study demonstrates that the transformed glial cell line RG-1 offers a sensitive self-reporting assay for GDNF expression.

Keywords *Renilla* luciferase · Reporter · Bioluminescence

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H. Liu · A.A. Szalay (✉)
Department of Biochemistry, Loma Linda University School of Medicine, Loma Linda, CA 92350, USA
E-mail: aszalay@som.llu.edu
Tel.: +1-909-8244300 Ext. 81360
Fax: +1-909-5580452

H. Liu
Department of Physiology and Pharmacology, Loma Linda University School of Medicine, Loma Linda, CA 92350, USA

R.P. Iacono
Department of Neurosurgery, Loma Linda University School of Medicine, Loma Linda, CA 92350, USA

Introduction

The primary pathology of idiopathic Parkinson's disease (PD) is linked to progressive loss of central monoamines, including dopamine (DA)-containing neurons mainly in the substantia nigra (Hornykiewicz 1973a, 1973b), as well as serotonin (5-HT) neurons (H. Liu et al. 1999). Supplementation with L-dopa has been the most effective therapy for alleviating the symptoms of PD, but it does not halt the progressive degeneration of nigral dopaminergic neurons (Ahlskog 1994). New approaches aim to provide protection to the DA neurons, and the most effective neuroprotection attained in animals to date has been achieved using glial cell line-derived neurotrophic factor (GDNF). GDNF was purified from conditioned medium from the rat glioma cell line B49, on the basis of its ability to promote high-affinity uptake of DA in cultures of embryonic day-16 rat ventral mesencephalon (Lin et al. 1993). The wild-type GDNF is a glycosylated homodimer; the monomer has an apparent molecular weight of 35 kDa on SDS-PAGE (Lin et al. 1994).

Although it remains to be demonstrated that a deficiency of GDNF is linked to PD, the trophic effect of nigral glia on DA cells in vitro (O'Malley et al. 1991) supports the hypothesis that degenerative disorders such as PD result from decreased availability of, or access or responsiveness to, specific survival factors. Numerous studies have demonstrated that GDNF may prevent nigrostriatal degeneration and actually reverse the behavioral deficits associated with nigrostriatal degeneration by enhancing the function of remaining dopaminergic neurons (Bohn 1999). These studies have indicated that GDNF has great potential as a useful therapy for Parkinson's disease.

A large number of trials of ex vivo GDNF gene therapy have been conducted in animals (Arenas et al. 1995; Lindner et al. 1995; Lapchak 1996, 1998; Perez et al. 1996). While the majority of these studies yielded very encouraging results that clearly demonstrated profound neuroprotective effects of GDNF, they could not monitor the viability and functionality of the implanted cells in vivo, since the monitoring must be done by postmortem biopsy. In these studies, the expression of a given protein in body fluids, i.e., cerebrospinal fluid, was determined by Western blotting and/or ELISA, the latter being the most sensitive and specific method available for quantifying GDNF produced by implanted cells. The detection limit of ELISA using a commercially available kit (GDNF E_{max} ImmunoAssay System; Promega, Madison, Wis.) is 32 pg/ml, while the concentration of GDNF required in culture to support survival of DA neurons is approximately 10–10,000 pg/ml (Bohn 1999). Because dilution occurs once a protein diffuses from the tissue matrix into cerebrospinal fluid, which is the only specimen that can be used to monitor implanted cells for GDNF production, it is obvious that ELISA is not sensitive enough to allow monitoring of the CSF for the GDNF produced by implanted cells.

To overcome the limits on detection of GDNF at very low concentrations, a more sensitive measure such as a reporter system is therefore highly desirable. Amongst the available reporter systems, *Renilla* luciferase was chosen for its (1) high sensitivity and low background in mammalian cells and body fluids, particularly CSF; (2) simplicity of detection and quantification; (3) lack of reported toxicity to mammalian cells; and (4) relatively low cost. This is an enzyme originating from the sea pansy *Renilla reniformis*, which catalyzes reactions that result in the emission of blue-green bioluminescence (Matthews et al. 1977). The monomeric *Renilla* luciferase protein, with a molecular weight of 36 kDa, catalyzes the oxidative decarboxylation of the complex organic molecule coelenterazine in the presence of dissolved oxygen, to yield oxyluciferin, CO_2, and blue light (λmax = 480 nm) in vitro (Mathews et al. 1977; Lorenz et al. 1996). *Renilla* luciferase has been used as a marker for gene expression in bacteria, as well as in plant and mammalian cells. It has also been fused to GFP to study energy transfer from *Renilla* luciferase to GFP (Wang and Szalay 1998), and the secreted form RUC fused to GFP can also be used to study the secretion of proteins (i.e., GFP) from transformed mammalian cells (J. Liu et al. 1999). These studies suggest that RUC in the form of a fusion protein can still retain its full enzymatic activity, and can thus be used as a reporter for the fused target protein, which also retains its activity.

In this report, we describe the establishment of a mammalian cell line that expresses and secretes a RUC-GDNF fusion protein. We demonstrate that the expression of GDNF can be monitored using the sensitive RUC bioluminescence assay when this cell line is implanted into the brain of the rat. We suggest that with further modifications this cell line could have great potential in gene therapy for Parkinson's disease, as well as other applications in basic and clinical research.

Materials and methods

Plasmid and phagemid vectors

The phagemid construct pGDNF3a, containing the rat GDNF cDNA with the signal peptide sequence, was generously provided by Dr. Martha Bohn (Northwestern University, Chicago, Ill.). Plasmid pC-RUC-BP6 encoding the secreted form of RUC (sRUC) was a gift from Dr. Yubao Wang (Wang et al. 2001). The human interleukin-2 (IL-2) signal peptide sequence was fused to the N-terminus of *Renilla* luciferase in a previous study to make this secreted form of RUC (Liu et al. 1997). The plasmid pBabe-PURO was kindly provided by Dr. Karoly Fatyol in our laboratory, and used to subclone the puromycin-N-acetyl-transferase (*pac*) gene. The plasmid pLNCX (6.6 kb) was purchased from Clontech (Palo Alto, Calif.), and was used as the backbone vector for all mammalian expression constructs used in this study.

Mammalian cell lines

The PO-D17 cell line was kindly supplied by Dr. Phyllis S. Frisa (Case Western Reserve University, Cleveland, Ohio). It was prepared from neonatal mouse cortical astrocytes immortalized by retrovirus-mediated transfer of the SV40 large T antigen gene (Frisa et al. 1996). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker, Walkersville, Md.), supplemented with 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, Md.), at 37°C in 5% CO_2.

Animals

Male adult Sprague-Dawley rats (Simonson Laboratories, Gilroy, Calif.) weighing approximately 200 g were used for cell implantations, and their cerebrospinal fluid was subsequently examined for bioluminescence. The animals were housed individually before and after surgery. Pregnant adult sheep were obtained from Nebeker Ranch (Lancaster, Calif.) and near-term (140–143 days) fetal sheep were used to harvest dorsal root ganglia (DRG).

Construction of vectors expressing RUC-GDNF

The plasmid pLNCX-sRUC-GDNF (Fig. 1A), which carries a RUC-GDNF expression cassette, was produced by inserting the cDNA for *Renilla* luciferase (secreted form, sRUC), fused to a GDNF cDNA, into the expression vector pLCNX.

The DNA fragment encoding sRUC was amplified by PCR from pC-RUC-BP6, using Primer-1 and Primer-2 (Table 1) and thus removing the stop codon and adding a *Hin*dIII restriction site at the 5′ end and an *Xho*I site at the 3′ end of the sRUC-encoding DNA, to facilitate subcloning. PCR was carried out with Deep Vent DNA polymerase (New England Biolabs, Beverly, Mass.) under the following conditions: hot start at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min.

Primer-3 and Primer-4 (Table 1) were designed to amplify from pGDNF3a a GDNF cDNA that did not include the coding sequence for the signal peptide. These primers were also designed to add an *Xho*I site at the 5′ end and a *Sal*I site at the 3′ end of the GDNF cDNA to facilitate subcloning. The PCR conditions used were: hot start at 94°C for 2 min, then 30 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 2 min.

The resulting PCR products, a 592-bp fragment which encodes GDNF, and a 1024-bp fragment encoding the sRUC with the

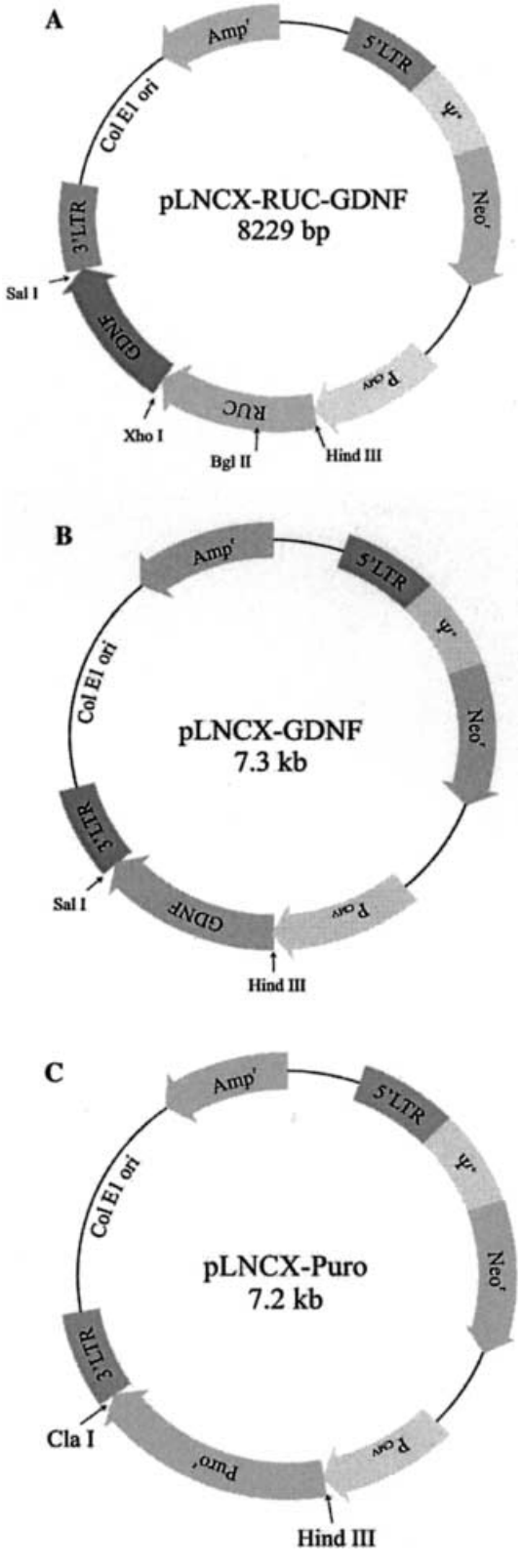

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Fig. 1A–C The *ruc-gdnf* fusion gene construct. **A** The expression vector pLNCX-sRUC-GDNF (*sruc* comprises the sequence encoding the human interleukin-2 signal peptide placed upstream of the RUC cDNA). **B** The intermediary vector pLNCX-GDNF was constructed by ligating a GDNF cDNA, excised from pGDNF3a, to pLNCX, in order to provide a *Sal*I restriction site necessary for the construction of pLNCX-sRUC-GDNF. The vector pLNCX-PURO (C) was used, together with pLNCX-sRUC-GDNF, for co-transformation of mammalian cells, to provide resistance to puromycin, as a selection marker

Table 1 List of primers used in subcloning and sequencing

Primer	Use	Sequence (5′→3′)
Primer-1	*ruc* subcloning	TTTAAGCTTATGTA-CAGGATGCAACTCCT
Primer-2	*ruc* subcloning	CGTCTCGAGCTTGTT-CATTTTTGAGAACTCGC
Primer-3	GDNF subcloning	TTTCTCGAGTTCCCGCTGC-CCGC
Primer-4	GDNF subcloning	GCGCGTCGACCAGGGT CAGATACATCCAC
Primer-5	Sequencing	CGCAAGAAGATGCACCTG
Primer-6	Sequencing	CATTCCCCCCTTTTTCTG

signal peptide, were gel-purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, Calif.). The purified DNAs was then digested with *Xho*I and *Sal*I (*gdnf*) and *Hin*dIII and *Xho*I (*sruc*) overnight at 37°C.

An intermediate construct pLNCX-GDNF (Fig. 1B), was produced by digesting pGDNF3a with *Hin*dIII and *Sal*I, followed by blunt-end ligation of the excised fragment into pLNCX, which had previously been digested with *Hpa*I and dephosphorylated. The sole purpose of pLNCX-gdnf was to modify the vector pLNCX by adding a *Sal*I restriction site. This vector was linearized by digestion with *Hin*dIII and *Sal*I, and gel-purified. The linearized pLNCX was then ligated with the sRUC and GDNF-encoding fragments.

To allow us to select for plasmid maintenance in mammalian cells, pLNCX-PURO (Fig. 1C), carrying a puromycin resistance gene (*puro*^{r}), was generated by inserting puro^r DNA into the MCS region of pLNCX. The full-length PURO^r DNA was obtained from pBabe-PURO by digestion with *Hin*dIII and *Cla*I, and inserted into the corresponding sites in pLNCX. This construct was co-transformed with pLNCX-sRUC-GDNF for drug selection purposes.

Establishment of permanently transformed mammalian cell lines

The cell line PO-D17 was co-transformed with pLNCX-sRUC-GDNF and pLNCX-PURO in 1:1 molar ratio. The FuGene6 transformation reagent (Roche Diagnostics, Mannheim, Germany) was used to carry out the transformation. The vector pLNCX was used as the control.

Cells transformed with pLNCX-sRUC-GDNF, or pLNCX, were exposed to puromycin selection for isolation of permanently transformed cells. The initial dose of puromycin (1 μg/ml) was included in the culture medium (DMEM with 10% FBS) for approximately 72–96 h, until defined foci had formed. These single clones were then transferred to 24-well tissue culture plates, and the concentration of puromycin was gradually increased by 0.5 μg/ml every 48 h, until a level of 5 μg/ml was reached and then maintained. The pLNCX-sRUC-GDNF-transformed clone of PO-D17 that displayed the highest RUC activity in the medium was chosen for stereotactic implantation and named RG-1. To examine the stability of fusion protein production in the transformed cells, the RUC bioassay was performed 24 h after the addition of 0.5 mg/ml puromycin, and thereafter at 48 h, 72 h, 1 week, and 2 months.

RUC bioassay and the spectrum of luminescence

RG-1 cells were grown to a monolayer in DMEM supplemented with 10% FBS in 60-mm culture plates. The cells were then gently

washed twice with ice-cold PBS, and cultured in serum-free DMEM for a further 24 h. To harvest medium and cells, the dishes were removed from the incubator and placed on crushed ice. Culture medium (3 ml) was collected by centrifugation at 600×*g* and 4°C for 5 min to pellet the cells. The supernatant was then concentrated tenfold to approximately 300 µl by filtration through the Amicon Centriplus concentrator-100 (Millipore, Bedford, Mass.) and stored at –70°C.

The attached cells from the same dishes were washed twice with ice-cold PBS, followed by the addition of 0.5 ml of ice-cold lysis buffer (50 mM TRIS-HCl pH 8.0, 150 mM NaCl, 0.02% sodium azide, 100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1% Triton X-100). After incubation on ice for 20 min, the cell layer was scraped off with a rubber policeman. The cell debris was transferred to a chilled microfuge tube and was homogenized by sonication. The homogenate was centrifuged at 12,000×*g* for 2 min at 4°C. The supernatant (approximately 600 µl) was transferred to a fresh microfuge tube, and stored in –70°C.

To determine the luciferase enzyme activity, an equal percentage of the cellular and medium fraction (1% of the total) was mixed with 100 µl of 0.5 µM coelenterazine, and light emission was measured using a Turner TD-20e Luminometer (Turner Designs, Sunnyvale, Calif.) and the Hamamatsu Argus-100 low-light imaging system (Hamamatsu Photonics K.K., Shizuoka, Japan).

To independently verify luciferase activity by spectral analysis, about 1×10^7 transformed cells were mixed with 0.5 µM coelenterazine and loaded into the FluroMax-3 Spectrofluorometer (Jobin Yvon, Edison, N.J.). The spectrum of the light emission from transformed cells was compared to that from the purified *Renilla* luciferase enzyme as a control (λ_{max} 472 nm).

GDNF bioassay

To assay GDNF activity, cultures of dorsal root ganglion (DRG) from near-term fetal sheep were used. For each experiment, five dorsal root ganglia or superior cervical ganglia were dissected from lumbar segments of the spinal cord and the cells were dissociated by incubation in digestion buffer (DMEM, 1× antibiotics, 10 mg/ml trypsin, 2.2 mg/ml DNase, 2 mg/ml collagenase D) for 4 h at 37°C with intermittent mixing. The cells were pelleted by centrifugation at 600×*g* and 4°C for 5 min, re-suspended in DMEM supplemented with antibiotics, and placed on a 10-cm tissue culture dish (Corning Glass Works, Corning, N.Y.). After incubation for 4 h at 37°C under 5% CO_2, the cell culture medium was aspirated. Cells attached to the dish surface were mostly non-neuron cells and were discarded. The cells in the aspirated medium were then centrifuged down, replaced in DMEM with 10% FBS, and incubated for 8 h to allow the cells to attach to the plate.

After washing twice with ice-cold PBS, aliquots of DRG cell culture were treated with equal amounts of one of three extracts: purified *Renilla* luciferase in DMEM (Med-RUC), 24-h conditioned medium of RG-1 cells (Med-RG1) and medium from cells stably transformed with the pLNCX vector control (Med-mock). The amount of Med-RUC and Med-RG1 was standardized by measuring light emission, and the amount of Med-mock was standardized to that of Med-RG1 on the basis of total protein concentration. The cells were treated for 72 h and examined by light microscopy for the outgrowth of neurites. Neurites that were at least twice as long as the cell body were defined as mature.

Western analysis of fusion proteins

Western analysis was performed to determine the molecular weight of the fusion proteins in 10 µl of concentrated supernatant. The protein samples were boiled at 100°C for 3 min before fractionation by SDS-PAGE. SDS-PAGE was performed in a 12% separating gel using a 5% stacking gel, and the resolved proteins were electrophoretically transferred onto a nitrocellulose membrane (WSI, Westboro, Mass.). Immunoblot analysis was performed with a 1:3000 dilution of a polyclonal GDNF antibody (chicken anti-human GDNF, Promega), horseradish peroxidase-conjugated rabbit anti-chicken IgY (Promega), and Opti-4CN substrate (Bio-Rad, Hercules, Calif.) as detection reagents, according to the manufacturers' instructions.

GDNF quantitation

ELISA was performed to determine the concentration of GDNF in RG-1 culture medium, in order to establish the equimolar relationship between RUC activity/concentration and GDNF concentration. The procedure was based on the GDNF Emax Immunoassay System (Promega) and was carried out following the manufacturer's instructions.

Confirmation of integration of the *ruc-gdnf* fusion into genomic DNA

To confirm that the fusion construct pLNCX-sRUC-GDNF had integrated into the host (RG-1) genome, PCR was performed on the host genomic DNA. Genomic DNA was extracted from the transformed cells (RG-1 and the mock transformed cell line) by standard techniques (Sambrook et al. 1989). Oligonucleotide Primer-5 and Primer-6 (Table 1) were designed to amplify the sequences of *ruc* and rat *gdnf*, as well as the linker regions.

The PCR was carried out with 2 U of Deep Vent DNA polymerase for 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min. The extension time at 72°C was increased to 7 min in the last cycle. Amplified products were checked after electrophoresis on a 1% agarose gel, and the identity of the fusion gene sequences was then confirmed by DNA sequencing.

Stereotactic neurosurgery

Adult male Sprague-Dawley rats of approximately 200 g body weight were used to test for secretion of the RUC-GDNF fusion protein by the implanted cell line (RG-1) in live animals. RG-1 cells were injected into the brain, and at defined time points, CSF was aspirated and analyzed for RUC activity.

The target for the implantation was the caudate putamen (Fig. 2). The coordinates of the target were (1) anterior-posterior axis: 0.8 mm posterior to the bregma; (2) lateral: 3.0 mm to the midline; (3) dorsal-ventral axis: –4.5 mm. Before implantation, the animal was anesthetized with sodium pentobarbital (Nembutal) at 63 mg/kg body weight. The animal was then immobilized on the stereotactic head frame with bite bar and ear bars. After the landmarks, bregma and lambda, were localized, the coordinates for caudate-putamen were marked on the skull in order to drill the surgical hole which was approximately 2 mm in diameter. A 26S gauge Hamilton needle (VWR Scientific, Brisbane, Calif.) attached to a 10-µl Hamilton syringe was then placed over the surgical hole defined by the anterior-posterior and lateral axes. The needle was then slowly lowered to the target defined by the dorsal-ventral axis. Approximately 1×10^6 RG-1 cells suspended in 10 µl of DMEM were injected into the target tissue at a rate of 0.5 µl/min. After injection, the surgical hole was closed by suturing the scalp, and the animal was returned to an individual cage supplied with food and water.

Before cell injection, a base-line sample of CSF (15–20 µl) was drawn from the cisterna magna with a 20-µl Hamilton syringe using a 27 gauge needle. The needle was positioned 45° along the midline with the bevel facing posteriorly. Extreme caution was taken when inserting the needle to avoid damage to the medulla rostral to the cistern. During the experiment, multiple cisternal CSF samplings were performed at 6 h, 1, 2, 3, 5, 10, and 15 days post implantation. The *Renilla* luciferase assay was carried out using 10 µl of cisternal CSF collected at different time points.

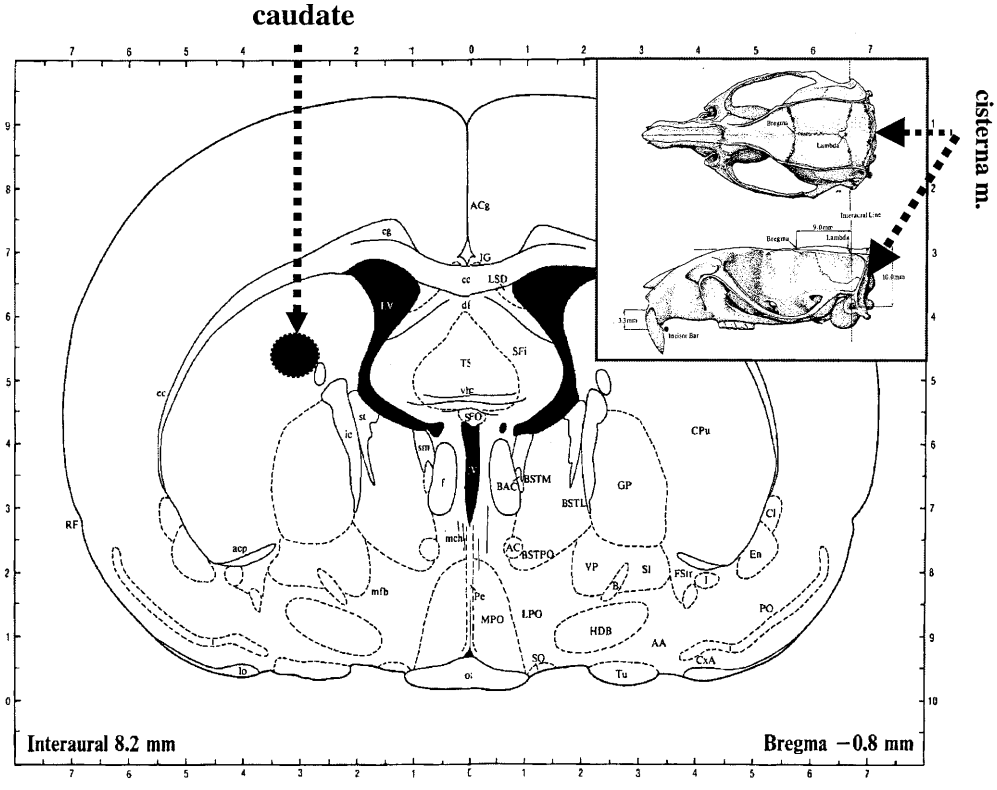

Fig. 2 Stereotactic implantations: targeting. The caudate is the target of cell implantation, the cistern is the source of CSF. The *ticks* indicate mm. The *filled circle* indicates the target (Caudate). The shaded regions are lateral and third ventricles. The CSF then circulates out of the fourth ventricle to the cisterna magna where it is sampled. The *inset* shows the location of the cistern

Results

Verification of the structure of the fusion constructs by DNA sequencing and Western analysis

Determination of the DNA sequences spanning the linker and its adjacent regions in the pLNCX-sRUC-GDNF fusion construct revealed that the downstream coding sequences were in fact fused in-frame. The sequence obtained from pLNCX-sRUC-GDNF DNA contained several point mutations in the 3′-terminal region of *ruc* and the linker region (not shown). These mutations, however, did not result in frame shifts in the GDNF coding sequence.

Furthermore, the sequence of the PCR product generated with primers specific for the insert region, and obtained using genomic DNA from the RG-1 cell line, confirmed the presence of both *ruc* and *gdnf* sequences. This finding indicates that the *ruc-gdnf* fusion gene was integrated into the host genome and expression of the fusion protein by RG-1 cells could therefore be expected.

The expression and secretion of the fusion protein RUC-GDNF was confirmed by Western analysis of the culture medium taken from pLNCX-sRUC-GDNF transformed cells (RG-1), which showed a single band of 70–75 kDa when incubated with anti-human GDNF antibody (Fig. 3). This molecular weight matches the predicted size for the fusion protein.

The fusion protein RUC-GDNF retains both RUC and GDNF activities

Luciferase activity was detected 24 h after transformation of PO-D17 cells with the DNA construct pLNCX-sRUC-GDNF. In addition, the maximum light emission in the cell lysate of RG-1 was at 472 nm, matching the λmax of the purified RUC protein (Fig. 4), suggesting that the light emission from the RG-1 cell lysate was indeed mediated by RUC which is part of the fusion protein RUC-GDNF.

That the fusion protein RUC-GDNF retained GDNF activity was confirmed by the fetal sheep DRG assay (Fig. 5). This experiment demonstrated that the conditioned culture medium of RG-1 containing the secreted RUC-GDNF fusion proteins significantly promoted DRG neurite outgrowth. In contrast, medium used to culture cells transformed with the GDNF(–) plasmid, and the purified RUC protein, showed no such effect.

Expression pattern of the fusion protein in RG-1 cell line

In DMEM supplemented with 10% FBS, the doubling time for the RG-1 cell line was about 48 h. Morphology and growth rate remained stable in this medium. Contact inhibition was retained but reduced compared to the non-transformed PO-D17 cells.

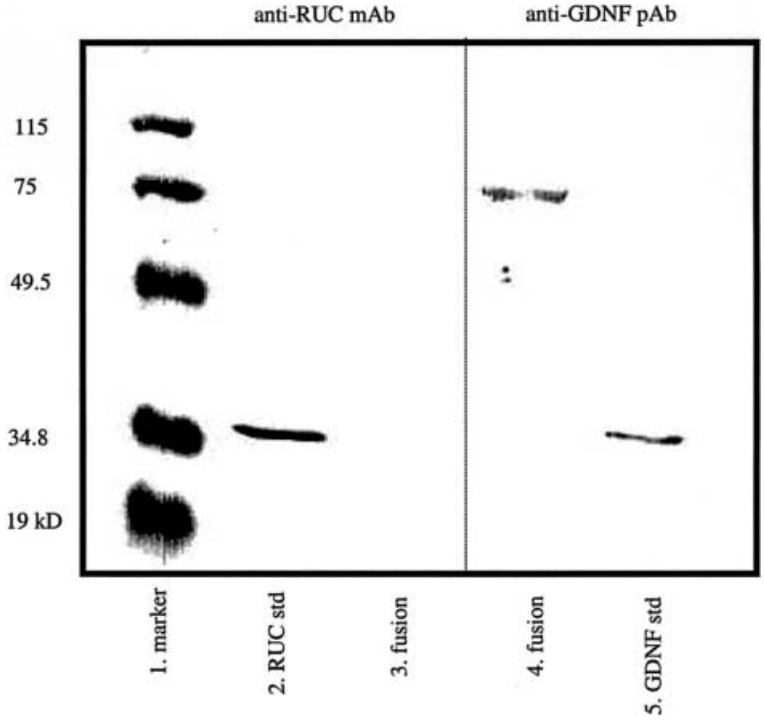

Fig. 3 Western analysis of proteins secreted into the culture medium. The lanes contained: 1, protein markers; 2, purified RUC protein; 3 and 4, RG-1 culture medium (fusion protein); 5, purified GDNF protein. Lanes 2 and 3 were probed with anti-RUC mAb, lanes 4 and 5 with anti-GDNF pAb. Purified RUC enzyme shows an apparent molecular weight of about 35 kDa, GDNF approximately 33 kDa. The fusion protein recognized by the GDNF-specific antibody has a molecular weight of approximately 72 kDa, while the anti-RUC antibody failed to recognize the fusion protein. The separating gel contained 12% acrylamide, and lanes 3 and 4 were loaded with 30-µl aliquots of 100-fold concentrated serum-free culture medium

The RG-1 cell line remained viable and functional in terms of RUC-GDNF secretion in a completely confluent monolayer for at least 2 weeks without passaging. Cultures of RG-1 exhibited a high level of the RUC activity in medium and low levels in cell lysate. This high secretability of the fusion protein is a highly desirable property for gene therapy applications of this cell line. RG-1 cells produced and secreted the RUC-GDNF fusion protein over a 2-month period, and in the presence of the maintenance dose of puromycin (5 µg/ml) in the culture medium, no decline in RUC-GDNF secretion was found. RUC activity readings obtained from culture medium of RG-1 cell lines remained at 5×10^6 photons/3 min/100 µl (n = 4, S.D. = 1.2×10^5) during the 2-month culture period.

RUC activity correlates with GDNF concentration in the RG-1 cell culture

The concentration of GDNF in the culture medium of RG-1 was determined by ELISA, and found to correlates with the photon counts obtained in the RUC bioassay measured in the Hamamatsu Argus-100 low-light imager. Figure 6 shows that the correlation coefficiency is 0.964 with $p < 0.01$, suggesting that luciferase activity in the culture medium accurately reflects the concentration of GDNF in the same culture.

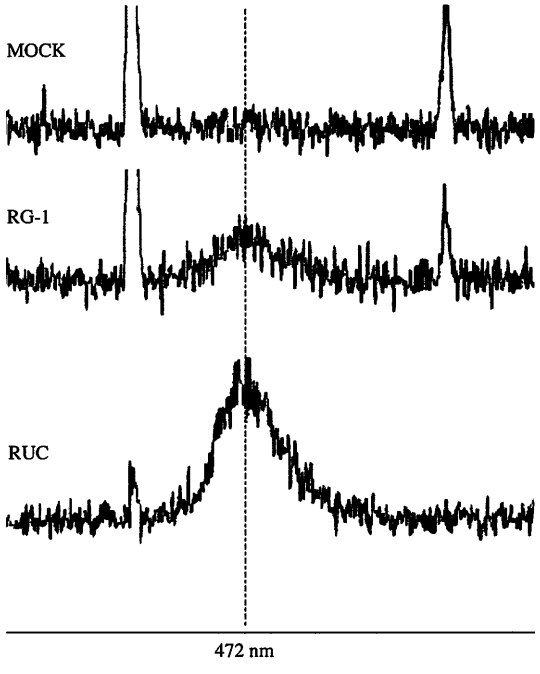

Fig. 4 Light emission spectra of RUC-GDNF fusion proteins. The maximum light emission from RG-1 cell lysates occurs at 472 nm (marked by the *dotted line*), the same as that of purified RUC. This indicates that the light emission of the reaction is indeed mediated by the RUC portion of the RUC-GDNF fusion

Renilla luciferase activity reports RUC-GDNF production in vivo

Figure 7 shows the luminescence in 10 µl of a cisternal CSF sample taken from rats before implantation (base line) and at 24 and 48 h, 5, 7, 10, 15, 20 and 25 days after implantation. RUC activity can be detected 24 h post-implantation and continues to rise at 48 h. RUC activity gradually declined at day 5, and was back to the base line by day 15.

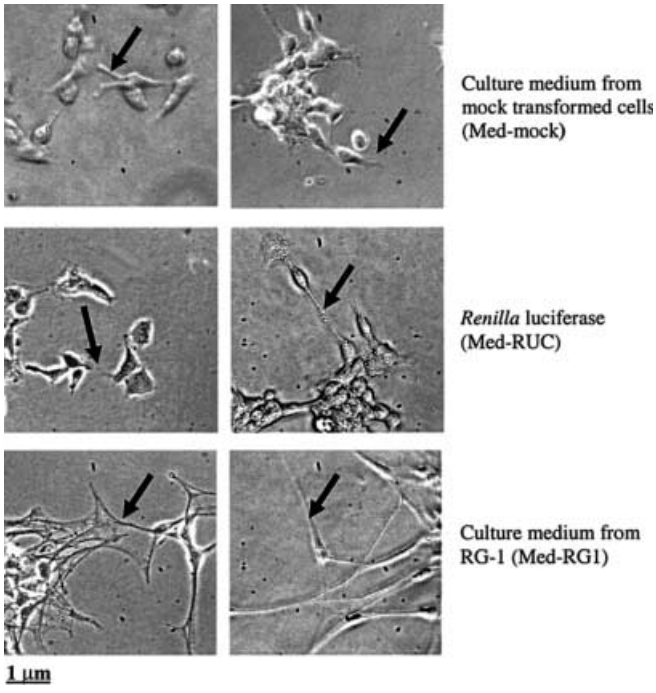

Fig. 5 GDNF bioassay using sheep DRG. DRG cultures were treated with the following three media for 72 h: Med-mock, mock control medium; Med-RUC, purified RUC enzyme solution; Med-RG1, RG-1 cell culture medium. The volumes of Med-RUC and Med-RG1 used were normalized by RUG photon counts. Images are from the light microscope at 30X. Neurites are indicated by the *arrows*. Compared to controls (medium from cultures of mock transformed cells and purified RUC), the RG-1 medium promoted significant neurite outgrowth

Five day-3 CSF samples of up to 15 μl volume were tested for the presence of GDNF using ELISA and Western blotting. Under experimental conditions much the same as those used in the experiments with RG-1 culture medium, no GDNF could be detected in CSF. No obvious neurite outgrowth could be seen in DRG cell cultures treated with 10 μl CSF obtained 72 h post-implantation.

Discussion

One of the limitations of current ex vivo gene therapy is the lack of a monitoring system that can measure the level of therapeutic product delivered. The purpose of this study was to establish an intrinsic reporter system to be used to monitor the expression levels of a transgene and, indirectly, the function of transformed and implanted cells. In this report, we have described the engineering of a mammalian cell line (RG-1) which was transformed with a GDNF-RUC fusion gene construct. The expressed fusion protein retains both RUC and GDNF activity, and cell implantation yields detectable RUC activity that can be used to monitor production of GDNF by RG-1 cells in live animals by assaying CSF samples.

The fusion protein RUC-GDNF secreted by the RG-1 cell line is as active as GDNF, as demonstrated by the GDNF bioassay using DRG cells. DRG cells are tyrosine hydroxylase $(TH)^+$ cells and have been commonly used as the cell culture model to test for GDNF activity.

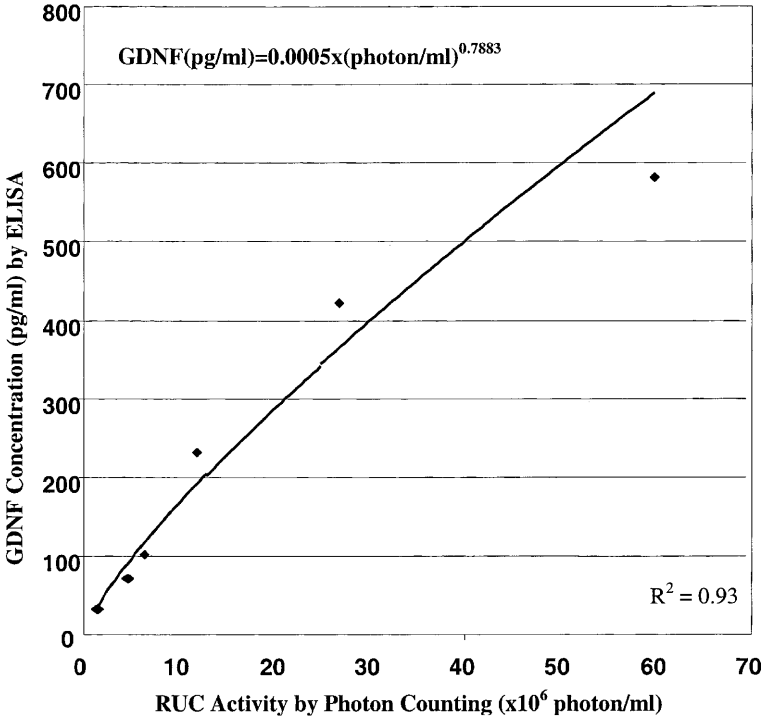

Fig. 6 Levels of GDNF determined by ELISA correlate with RUC activity. The correlation between photon counts and GDNF concentration is reflected in the R^2 value (0.93), and the regression is described by the equation. The graph suggests that GDNF and RUC are expressed in an approximately equimolar ratio, and that the RUC activity assay can be used to estimate GDNF production in the culture medium

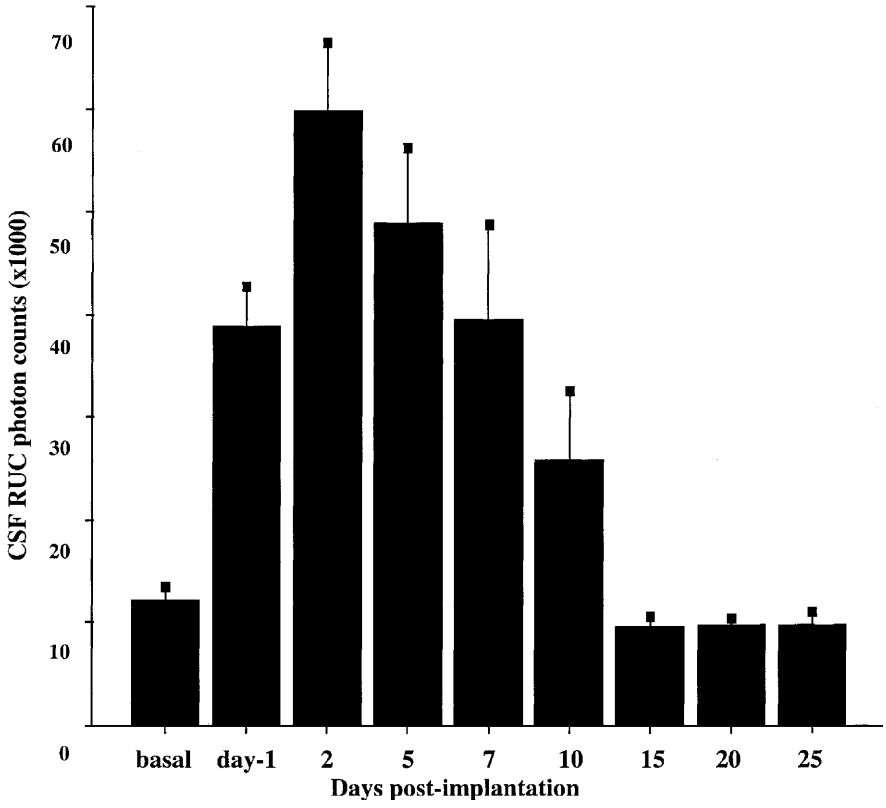

Fig. 7 Detection of secreted RUC activity in the cisternal CSF at various times after implantation of RG-1 cells. RUC activity (photon counts) is plotted against time after implantation. Each bar represents a single time point. Approximately equal numbers (1×10^6) of RG-1 cells were implanted in each of 17 rats. All animals consistently show that RUC activity peaked at day-2 in this plot. However, activity could actually have peaked on day-3, when the CSF samples were used for GDNF assays. By day-15, RUC activity had declined to base line. The volume of CSF sample used was 10 μl

These cells express the GDNF receptors identified to date – RET, GFR alpha-1, and GFR alpha-2 (Molliver et al. 1997; Bennett et al. 1998; Kashiba et al. 1998). The neurotoxin 6-OHDA, which is selective for dopaminergic cells, could also be used to test for the effects of GDNF on cell survival. Morphological changes in DRG neurons after treatment with RG-1 medium are clear-cut, i.e., neurite outgrowth. Therefore further confirmation with TH immunocytochemical staining is not necessary.

The correlation between RUC-dependent photon counts and GDNF concentration as determined by ELISA was found to be very high ($R^2=0.93$, Fig. 6); however, the relationship is non-linear. The reason for the non-linearity is that the photon counting was done with the Hamamatsu micro-channel Argus-100 plate camera, which is known to have a narrow linearity range. The high correlation strongly suggests that RUC-GDNF is synthesized as a fusion protein, secreted as a fusion protein, and circulates in the CSF as a fusion protein.

The RUC reporter system has high sensitivity, which is reflected in the contrast between the positive RUC bioluminescence assay and the always negative GDNF assays in CSF. This finding further demonstrates that RUC as the reporter offers sensitivity beyond the detection limits of the commonly used protein determination methods, such as ELISA and Western blotting.

The expression levels of RUC-GDNF protein in the RG-1 cell line are relatively high. The stability of fusion gene expression in the host genome was facilitated by the retention of the CMV promoter (P_{CMV}), demonstrated by PCR. Even though P_{CMV} drives strong expression of the transgene, stronger and cell-specific promoters can be used to optimize the expression of these fusion proteins. One additional candidate promoter is the GFAP (glial fibrillary acidic protein) promoter, which is active in the glial cell line PO-D17. GFAP promoter-driven expression of the transgene may offer cell specific expression and tight regulation in glial cells (Morelli et al. 1999). The expression level may also be significantly improved by utilizing an amplification-promoting element, such as muNTS1, to increase the copy number of the integrated DNA by up to 800-fold (Hemann et al. 1994). Use of adenovirus-mediated gene transfer may also improve the expression efficiency (Barkats et al. 1998).

We have found that the RUC-GDNF fusion protein activity in CSF declined after 5 days of implantation. The explanation for the decline could be that the implanted cells are rejected by the host immune system. Another explanation may be that the expression of fusion gene is switched off by silencing. Immune response reactions in the brain are limited, compared to the rest of the body, partly due to the blood brain barrier. However, brain immune responses against cellular transplants have been observed in mice (Terry et al. 1997); the histological changes observed post-implantation were similar to those seen in graft rejection.

Several other issues arise from our study that warrant further discussion. First, the sequence analysis of the fusion gene *ruc-gdnf* indicated several point mutations that did not result in frameshifts. In-frame translation of

pLNCX-sRUC-GDNF was demonstrated by Western analysis, which shows that the fusion protein RUC-GDNF is recognized by anti-GDNF antibody. Since the fusion protein RUC-GDNF is fully active as RUC and GDNF, no attempts were made to correct any of the point mutations. Spontaneous mutations have been reported to occur during the process of transformation (Bourre et al. 1991; Madzak et al. 1992).

Secondly, RG-1 demonstrated contact inhibition, which is a desirable property in cells destined for implantation, simply because immortalized cells with contact inhibition should not grow into tumors once implanted. This is an important criterion if encapsulation is used for cell implantation to protect implanted cells against immune rejection by the host. Implanted microcapsules may rupture and the cells may overgrow (Leu et al. 1992; Matthew et al. 1993). To protect the implant recipient from such an overgrowth, the introduction of an externally activatable "death switch" construct may be necessary. The apoptosis cascade could be induced by switching on a promoter, so that the number of implanted cells can be maintained at the therapeutic "dose".

To our surprise, the fusion protein RUC-GDNF, which clearly demonstrated RUC activity with the luminescence spectrum characteristic for *Renilla* luciferase, was not recognized by anti-RUC antibodies. The failure to recognize the *Renilla* luciferase component of the fusion might be due to a conformational change in the RUC portion that hinders the binding of the antibody. In contrast, the fusion does not prevent the binding of GDNF antibody.

Potential applications of the RG-1 cell line to express the fusion protein and to monitor cell survival, function, and implantation are important. First, it has been shown to be able to secrete functional RUC-GDNF fusion protein and report the secretion. We expect that the implanted cells expressing biologically active GDNF will offer protection to dopaminergic neurons and may even reverse the progression of PD in patients. Such cell lines may help researchers and clinicians to monitor the longevity of the implanted cells by a simple lumbar tap to measure RUC activity in CSF. The self-reporting feature offered by this cell system would also enable the researcher to study the relationship between implanted cells and the relief of PD symptoms and disease progress. In the past this was not possible because cells could not be monitored in the brain of live animals.

Furthermore, PD is not the only disease that could benefited from the RG-1 cell line. A recent study using DRG cells has demonstrated a potent analgesic effect of GDNF in neuropathic pain states (Boucher et al. 2000). Intrathecal implantation of the RG-1 cell line may alleviate severe and refractory pain due to peripheral nerve damage by providing a constant supply of GDNF.

Thirdly, the fusion protein from the RG-1 cell line may also be used in GDNF receptor studies. When the ligand, GDNF, that is fused to RUC, binds to the GDNF receptor, the concentration of receptor can be titrated in cell cultures by adding fusion protein to cells, then adding coelecterazine to the reaction and quantifying the light emission.

Lastly, the findings described in this study may also apply to gene therapy with other therapeutic recombinant proteins, such as amino acid decarboxylase (AADC) to enhance dopamine synthesis in the treatment of PD, and brain-derived neurotrophic factor (BDNF) to promote cholinergic neuron survival in the treatment of Huntington's disease. In general, as with the fusion protein RUC-GDNF, these proteins may also be fused with RUC as a reporter protein to achieve the intrinsic monitoring capability. We expect that this protein fusion technique will be widely used to engineer proteins with therapeutic functions for cellular implantation.

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