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TRANSPLANTATION OF FETAL VENTRAL MESENCEPHALIC PROGENITOR CELLS OVEREXPRESSING HIGH MOLECULAR WEIGHT FIBROBLAST GROWTH FACTOR 2 ISOFORMS IN 6-HYDROXYDOPAMINE LESIONED RATS

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Abstract—Fibroblast growth factor-2 (FGF-2) is a potent neurotrophic factor promoting survival of dopaminergic (DA) neurons *in vitro* and *in vivo*. FGF-2 is expressed in different isoforms representing distinct translation products from a single mRNA. For this study, we focused on the high molecular weight (HMW) isoform, which, after non-viral plasmid-based overexpression in embryonic day 12 (E12) rat ventral mesencephalon (VM)-derived cells, revealed increased numbers of tyrosine hydroxylase-positive (TH^+) cells in a 'colayer' cell culture model. To determine the therapeutic potential of VM cells producing FGF-2-HMW as their 'own' neurotrophic factor, we transplanted cell suspensions obtained from such *in vitro* modified and differentiated cell cultures into the 6-hydroxydopamine (6-OHDA) hemiparkinsonian rat model. Animals, having received either non-transfected cells, empty-control transfected, or FGF-2-HMW-plasmid transfected cells, were analyzed in two different transplantation paradigms each using 172,000 or 520,000 cells, respectively. The behavioral performances in the amphetamine- and apomorphine-induced rotational test as well as in the cylinder test were evaluated for up to thirteen weeks post transplantation (postTX). Finally, the integration of the grafted cells into the host striatum was analyzed by immunohistochemical measurements. Those analyses revealed improvements of behavioral deficits in all five groups receiving DA neuron grafts, except for amphetamine-induced rotation of the FGF-2-HMW small graft group. Altogether, genetic modification with the FGF-2-HMW-plasmid did not further improve functional recovery compared to the control groups and had no influence on either the number of surviving DA neurons or on the density of outgrowing TH^+ fibers.

Key words: brain repair, dopaminergic neuron, neurotrophic factor, nucleofection, Parkinson's disease, striatum.

INTRODUCTION

Parkinson's disease (PD) is one of the most common neurodegenerative disorders, caused by a progressive loss of dopaminergic (DA) neurons in the substantia nigra with resulting dopamine depletion in the striatum. To date, the gold standard in PD therapy is the symptomatic treatment of the motor symptoms by administration of L-DOPA, a dopamine precursor, or dopamine receptor agonists. Although drug therapy yields effective control over many of the symptoms, long-term treatment holds great risks of undesirable side-effects like L-DOPA-induced dyskinesia (Cenci and Lindgren, 2007) and, in addition, a 'wearing off' of the potency occurs. An alternative therapeutic strategy is the cell-based restorative approach where the missing dopamine is substituted by intrastriatally transplanted fetal DA neurons. Transplantation holds great promises, as beneficial effects have been observed in numerous animal experiments and open-label clinical studies (Björklund, 1992; Hauser et al., 1999; Lindvall and Hagell, 2000; Winkler et al., 2000; Mendez et al., 2008), although motor complications have been reported by two double-blind clinical trials (Freed et al., 2001; Olanow et al., 2003).

Critical factors determining the functionality of the graft are the number of DA neurons surviving the transplantation procedure, as they represent a very vulnerable cell-type, and their striatal innervation (Björklund et al., 1987; Brundin et al., 2000). One reason for the poor survival of transplanted cells could be the insufficient support by neurotrophic factors. Several factors have been reported to promote development,

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Abbreviations: 6-OHDA, 6-hydroxydopamine; ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; DA, dopaminergic; DMEM, Dulbecco's modified Eagle's medium; E12, embryonic day 12; FCS, fetal calf serum; FGF-2, fibroblast growth factor 2; GDNF, glial cell-derived neurotrophic factor; HMW, high molecular weight; LMW, low molecular weight; nt, non-transfected; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PD, Parkinson's disease; PFA, paraformaldehyde; postTX, post transplantation; preTX, prior to transplantation; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SEM, standard error of the mean; TH, tyrosine hydroxylase; TX, transplantation; VM, ventral mesencephalon.

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survival, and neurite outgrowth of mesencephalic DA neurons (Hyman et al., 1991; Beck, 1994; Rosenblad et al., 1996; Krieglstein, 2004). In addition to glial cell-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), fibroblast growth factor-2 (FGF-2) has been shown to support DA cells (Engele and Bohn, 1991; Mayer et al., 1993a; Grothe et al., 2000; Timmer et al., 2006; Grothe and Timmer, 2007).

Several studies have demonstrated that FGF-2 mediates survival- and neurite-promoting as well as neurotoxin-protecting effects on dissociated DA neurons and is involved in development and maintenance of mesencephalic DA neurons *in vitro* and *in vivo* (Beck, 1994; Grothe et al., 2000; Fontan et al., 2002; Grothe and Timmer, 2007; Timmer et al., 2007). The FGF-2 protein is expressed in different isoforms in the striatum and substantia nigra (Bean et al., 1991; Florkiewicz et al., 1991; Tooyama et al., 1992; Arese et al., 1999; Claus et al., 2004; Timmer et al., 2004) representing distinct translation products from a single mRNA, the low molecular weight (LMW) 18-kDa isoform and the high molecular weight (HMW) 21-kDa and 23-kDa isoforms, respectively. Both, LMW and HMW FGF-2 isoforms, have been shown to increase survival and fiber outgrowth of cultured DA neurons *in vitro* (Ferrari et al., 1989; Mayer et al., 1993a; Grothe et al., 2000; Jensen et al., 2008). *In vivo* studies showed that pretreatment of DA cell suspensions with FGF-2-LMW before transplantation resulted in an enhanced survival of tyrosine hydroxylase-positive (TH^+) cells after grafting (Mayer et al., 1993b). Moreover, after repeated intracerebral FGF-2-LMW infusions, the number of surviving grafted DA neurons increased further, which was accompanied by greater behavioral recovery (Mayer et al., 1993b). Another approach to provide intrastriatal DA grafts with FGF-2 was followed in co-transplantation attempts. Takayama et al. (1995) reported on an increased survival of DA neurons and an enhanced motor function after co-transplantation with FGF-2-LMW producing fibroblasts (Takayama et al., 1995). Furthermore, after co-transplantation of genetically modified Schwann cells over-expressing FGF-2 isoforms, FGF-2-HMW revealed better survival and striatal innervation as well as improved rotational behavior compared to FGF-2-LMW (Timmer et al., 2004).

To explore the effects of FGF-2-HMW on intrastriatal grafts devoid of foreign cell-types (e.g. Schwann cells), we utilized in this study our recently developed 'colayer' protocol for transfection of mesencephalic progenitor cells (Ratzka et al., 2012). We characterized the impact of FGF-2-HMW grafts compared to empty-control plasmid or non-transfected grafts with regard to DA neuron survival and striatal fiber integration and the effects on behavioral performance.

EXPERIMENTAL PROCEDURES

Expression plasmids and transfection

The coding sequence of the rat FGF-2 gene (NM_019305.2, GenBank) was amplified by polymerase chain reaction (PCR) with Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific, Schwerte, Germany) from plasmid pCI-neo-HMW, which carried a mutated FGF-2 sequence derived from the RSVpΔmetFGF vector (Pasumarthi et al., 1994; Claus et al., 2003). Namely, exchanges of the alternative CUG start codon of FGF-$2^{23\ kDa}$ to ATG and replacement of the FGF-$2^{18\ kDa}$ ATG start codon with a *Hin*dIII site. The primers used (RnFGF2_F GAATTCGCCGCCACCATGGCAGCCCGCGGGCGAGC, RnFGF2_R TCTAGAGCTCTTAGCAGACATTGGAAGA) removed the stop codon and added the underlined *Eco*RI- and *Xba*I-sites, respectively, to allow the transfer of FGF-2-HMW coding sequence into the pCAGGS-FLAG plasmid (Ratzka et al., 2012) in frame with a C-terminal 3xFLAG epitop resulting in pCAGGS-FGF-2-HMW-FLAG plasmid. The pCAGGS-FGF-2-LMW-FLAG and pCAGGS-empty-control plasmids have been described previously (Niwa et al., 1991; Ratzka et al., 2011).

For *in vitro* and *in vivo* experiments, pCAGGS plasmids were nucleofected with the Amaxa Nucleofector II device (Lonza, Cologne, Germany) using primary cells derived from the ventral mesencephalon (VM), which had been expanded in proliferation medium (composition see Section "Preparation of embryonic ventral mesencephalic tissue and cell culture") for three days. Cells were detached and 2,000,000 cells were re-suspended in 100 µl nucleofection solution and transfected with 5 µg of plasmid DNA using the Amaxa basic nucleofector kit for primary neurons (Lonza) and program A-033 as described previously (Cesnulevicius et al., 2006; Ratzka et al., 2012). After transfection and re-seeding, the cells were kept in differentiation medium (composition see Section "Preparation of embryonic ventral mesencephalic tissue and cell culture") for two (*in vivo*) or six days (*in vitro*), respectively. Non-transfected (nt) cells served as control.

Western blot

Six days after nucleofection with FGF-2-HMW, FGF-2-LMW, or empty-control plasmids, VM-derived cells (300,000 cells/well of a 6-well Nunc™ plate (Thermo Fisher Scientific)) were lysed in radioimmune precipitation assay buffer as described previously (Ratzka et al., 2012). From each sample 80 µg of protein were denatured by boiling in Laemmli buffer, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (12% gel) and transferred electrophoretically to Amersham Hybond ECL nitrocellulose membranes (GE Healthcare Life Sciences, Munich, Germany). The membranes were probed with mouse anti-FLAG-M2 antibody (F1804, Sigma–Aldrich, Munich, Germany, 1:3000), rabbit anti-β-Actin antibody (4967, Cell Signaling, Leiden, Netherlands, 1:1000) and secondary anti-mouse/rabbit antibodies conjugated to horseradish peroxidase (NA931V/NA934, GE Healthcare Life Sciences, 1:4000). By chemiluminescence reaction (Immobilon Western kit, Millipore, Darmstadt, Germany) protein bands were detected on a Chemiluminescence Imager system (Intas Science Imaging, Göttingen, Germany).

Preparation of embryonic ventral mesencephalic tissue and cell culture

VM-derived DA progenitor cells of embryonic day 12 (E12) rat embryos (crown-rump length of 6 mm) were