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Research Report

Dopaminergic differentiation of stem cells from human deciduous teeth and their therapeutic benefits for Parkinsonian rats

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ABSTRACT

Parkinson's disease (PD) is a progressive neurodegenerative disorder caused by the loss of nigrostriatal dopaminergic (DAergic) neurons and the depletion of striatal dopamine. Here we show that DAergic-neuron-like cells could be efficiently induced from stem cells derived from human exfoliated deciduous teeth (SHEDs), and that these induced cells had therapeutic benefits in a 6-OHDA-induced Parkinsonian rat model. In our protocol, EGF and bFGF signaling activated the SHED's expression of proneural genes, *Ngn2* and *Mash1*, and subsequent treatment with brain-derived neurotrophic factor (BDNF) promoted their maturation into DAergic neuron-like SHEDs (dSHEDs). A hypoxic DAergic differentiation protocol improved cell viability and enhanced the expression of multiple neurotrophic factors, including BDNF, GDNF, NT-3, and HGF. Engrafted dSHEDs survived in the striatum of Parkinsonian rats, improved the DA level more efficiently than engrafted undifferentiated SHEDs, and promoted the recovery from neurological deficits. Our findings further suggested that paracrine effects of dSHEDs contributed to neuroprotection against 6-OHDA-induced neurodegeneration and to nigrostriatal tract restoration. In addition, we found that the conditioned medium derived from dSHEDs protected primary neurons against 6-OHDA toxicity and accelerated neurite outgrowth in vitro. Thus, our data suggest that stem cells derived from dental pulp may have therapeutic benefits for PD.

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1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by extensive loss of dopaminergic (DAergic) neurons in the substantia nigra (SN) and ventral tegmental area (VTA). These losses lead to a striatal dopamine (DA) deficiency and a subsequent debilitating movement disorder (Braak et al., 2003; Dauer and Przedborski, 2003; German et al., 1989). Pharmacological treatment with the DA precursor L-dihydroxyphenylalanine palliates most early PD symptoms by replacing DA, but this treatment does not halt the degradation of DAergic neurons and is associated with severe side effects (Cummings, 1992; Fernandez-Espejo, 2004). An alternative approach, in which cells are replaced by transplanted human fetal mesencephalic tissues, ameliorates clinical symptoms in PD patients (Yamanaka et al., 2008a, 2008b) but faces major hurdles due to the limited availability of donor materials and the ethical concerns of using human fetal tissues (Freed et al., 1992; Lindvall et al., 1990; Olanow et al., 1996).

Stem cell-based transplantation therapy has been proposed as a promising approach to overcome the shortage of donor materials for treating PD. Factors that contribute to the generation of DAergic neurons during the embryonic period have been used to promote the DAergic neural differentiation of various types of stem cells (Neirinckx et al., 2013). DAergic neurons, which originate from the ventral part of the midbrain (Ang, 2006), are born where sonic hedgehog (SHH) signaling from the notochord intersects with FGF-8 from the isthmus (Hynes et al., 1995; Hynes and Rosenthal, 1999). TGF-β is essential for early SHH signaling and the subsequent induction of DAergic neurons (Farkas et al., 2003). These signals act on neuronal progenitors to specify anteroposterior and dorsoventral identity, and lead to the activation of multiple transcription factors required for the development of immature DAergic neurons (Ang, 2006; Jankovic et al., 2005). Glial-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) are also crucial for the survival and/or maturation of DAergic neurons (Baquet et al., 2005; Kramer et al., 2007; Li et al., 2012). These factors have been used alone or in combination to induce DAergic neurons from adult mesenchymal stem cells (MSCs) derived from bone marrow (BMSCs) (Hermann et al., 2004; BMSC; Trzaska et al., 2007), umbilical cord (Weiss et al., 2006), adipose tissue (McCoy et al., 2008), and dental pulp (DPSCs) (DPSC; Kanafi et al., 2014; Wang et al., 2010).

Human adult DPSCs and stem cells from human exfoliated deciduous teeth (SHEDs) are self-renewing MSCs that reside within the perivascular niche of the dental pulp (Gronthos et al., 2002). They are thought to originate from the cranial neural crest, which expresses early markers for both MSCs and neuroectodermal stem cells (Gronthos et al., 2000a; Miura et al., 2003b) and can differentiate into functional neurons (Gronthos et al., 2000a; Miura et al., 2003b) and oligodendrocytes (Sakai et al., 2012) under appropriate conditions. When engrafted, these dental pulp-derived stem cells promote functional recovery from various acute and chronic CNS insults through both cell replacement and paracrine mechanisms (Yamamoto et al., 2014; Young et al., 2013).

In this study, we analyzed the efficiency of SHED differentiation into DAergic neuron-like cells (dSHEDs) and the ability of dSHEDs to secrete DA. We also evaluated the therapeutic benefits of both SHEDs and dSHEDs when transplanted into 6-OHDA-induced Parkinsonian rats.

2. Results

2.1. *Differentiation of SHEDs into DAergic neuron-like cells*

The primary characteristics of the three primarily SHED lines used in this study are described in our previous report (Sakai et al., 2012). These SHEDs have a fibroblastic morphology with a bipolar spindle shape, and uniformly express adult BMSC markers (CD90, CD73, and CD105), neural stem/progenitor cell markers (Doublecortin, GFAP, and Nestin), and early neuronal and oligodendrocyte markers (βIII-Tubulin, A2B5, and CNPase), but not markers for mature oligodendrocytes (MBP and APC) (Sakai et al., 2012).

We examined the DAergic differentiation ability of these three SHED lines under four differentiation protocols, each with two steps, and observed all of these lines had similar differentiation activities. In the first step, neural preconditioning, the cells were seeded on gelatin-coated dishes with a medium containing bFGF and EGF. The SHEDs in step 1 were spindle-shaped (Fig. 1A). In the second step, DAergic differentiation, the medium was changed to basic induction medium (BIM) containing inductive agents. The highest proportion of TH-positive cells was obtained with a BDNF protocol (71.6±19.8%), compared to SHH and FGF-8 (31.7±9.6%), TGF-β (40.8±5.2%), GDNF protocols (51.0±14.07%) or BIM alone (26.1±2.6%) (Fig. 1C). We also examined whether the combinatorial stimulation of BDNF together with another inducer would accelerate the dopaminergic differentiation of SHEDs, but no synergistic effects were observed (data not shown). The BDNF-treated SHEDs exhibited a neuron-like morphology with multiple neurites (Fig. 1B), and expressed both TH and DA (Fig. 1F, G, J, K: dSHED). Statistical analysis showed that 91.1±6.4% of the β-Tubulin-positive cells expressed TH, and 78.8±15.2% of the β-Tubulin-positive cells expressed DA (Fig. 1L). The dSHEDs secreted more than 12 ng DA per 10^5 cells into the culture medium in 30 min after KCl-induced depolarization (Fig. 1M).

PCR analysis revealed elevated expressions of the proneural genes *Mash1* and *Ngn2* and the DA-inducer *Wnt1* in the SHEDs treated with EGF and bFGF in step 1 (Fig. 2A), while the expressions of other transcription factors and growth factors related to the differentiation/maturation of DAergic neurons were maintained throughout the induction. After step 2, the expression of proneural genes ended, but the DA-specific *Th* gene was activated. The neural maturation of dSHEDs, indicated by the expression of NeuN, which marks mature neurons, revealed that the BDNF protocol produced a significantly higher proportion of NeuN-positive cells (75.7±5.3%) than the other protocols. After step 2, most of the TH-positive dSHEDs co-expressed NeuN (Fig. 2C–F).

Although dSHEDs had a low survival rate (24.1±0.9%) at the end of step 2, we found that inducing dSHEDs under hypoxic conditions significantly improved their survival. The survival rate of dSHEDs cultured in 1 and 5% oxygen was 48.1±1.7%

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