Expression of the Parkinson’s Disease-Related Protein DJ-1 during Neural Stem Cell Proliferation

Hui YAN\textsuperscript{a,b} and Xiao-Ping Pu\textsuperscript{a,b}

\textsuperscript{a} State Key Research Laboratory of Natural and Biomimetic Drugs, Peking University; and \textsuperscript{b} Department of Molecular and Cellular Pharmacology, School of Pharmaceutical Science, Peking University; Beijing 100191, P. R. China.

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Neural stem cells (NSCs) arise through neurogenesis, and comprise all newly-formed neurons in the central and peripheral nervous systems. DJ-1 is associated with autosomal recessive familial Parkinson’s disease (PD). The relationship between DJ-1 and NSC proliferation may shed light on the underlying pathogenesis of, and potential treatments for, PD. To investigate the relationship between DJ-1 and NSCs, embryonic cortical NSCs were isolated and cultured from E14 fetal rats. Immunocytochemistry, flow cytometry, and immunohistochemistry were applied to evaluate DJ-1 expression in proliferating NSCs. We found that DJ-1 was co-expressed with nestin, a marker of progenitors, during NSC proliferation from days 1—7. The present results suggest that DJ-1 is co-expressed with nestin in NSCs during proliferation.

Key words neural stem cell; DJ-1; nestin; proliferation; Parkinson’s disease

Parkinson’s disease (PD) is characterized by a profound and selective loss of nigrostriatal dopaminergic neurons. Recent research has indicated that a combination of genetic susceptibilities and environmental factors critically contribute to the pathogenesis of PD. In addition, several genes are associated with sporadic PD, including \textalpha-synuclein, parkin, DJ-1, PINK-1, and LRRK2.\textsuperscript{1} DJ-1 is a 23 kDa protein that is expressed in peripheral tissues and selected brain regions.\textsuperscript{2} It has various functions, including oncogene activity, modulation of androgen-receptor-dependent transcription, and also acts as a sensor for oxidative stress.\textsuperscript{3—5} Overexpression of DJ-1 is neuroprotective, whereas mutations that inhibit its function lead to cell apoptosis or death\textsuperscript{5—7}; this latter effect is associated with a rare form of familial PD.

Neural stem cells (NSCs) are functionally defined as long-term, self-renewing cells that possess the ability to generate cells of the three major lineages of the nervous systems, including neurons, astrocytes and oligodendrocytes. Multipotent NSCs may be used as a cell-based therapy for neurological disorders, such as PD.\textsuperscript{8}

Because of the important role of NSCs in neurogenesis, greater insight into the relationship between DJ-1 and the development of NSCs may help explain the pathogenesis of PD and highlight ideas for novel PD therapies. In the present study, we isolated and cultured primary embryonic NSCs from the cortex of fetal rats and investigated the expression of DJ-1 in proliferating NSCs.

MATERIALS AND METHODS

Animals

Pregnant Sprague-Dawley rats were purchased from the Laboratory Animal Center of Peking University Health Science Center (Beijing, China) and delivered to our facility on gestational day 14. Animals were housed under standard conditions (temperature 22±2 °C, relative humidity 55±5%, 12-h-light/dark cycle) with food and water available \textit{ad libitum}. All experiments were performed under the guidelines of the Experimental Laboratory Animal Committee of Peking University Health Science Center and were in strict accordance with the principles and guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol was approved by the local animal committee with the confirmation number SCXK (Jing) 2002-0001.

Materials

Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (1 : 1) and Hank’s balanced salt solution (HBSS) were obtained from Hyclone (Logan, UT, U.S.A.). B27 supplement was purchased from Invitrogen Co. (Carlsbad, CA, U.S.A.). Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), poly-L-lysine, diethyl pyrocarbonate (DEPC) water and Hoechst 33342 were obtained from Sigma (St. Louis, MO, U.S.A.). Rabbit anti-nestin polyclonal antibody was obtained from Chemicon International Inc. (Temecula, CA, U.S.A.). Rabbit anti-DJ-1 polyclonal antibody was the kind gift of Prof. Hiroyoshi Ariga (Hokkaido University, Japan). Fluorescein isothiocyanate (FITC)-conjugated and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (CA, U.S.A.).

Cortical NSCs Culture

Primary cortical neural stem cells (NSCs) were cultured as previously described.\textsuperscript{9} At E14, the pregnant female rat was sacrificed and the intact embryo brains were removed. The cortex was dissected and placed in ice-cold HBSS. Cells were mechanically dissociated by trituration and plated at a density of 5×10⁴ cells/cm² (T-25 cm² flask, Corning, U.S.A.) or 5×10⁵ cells/cm² (96-well plates, BD, U.S.A.). Cells were expanded for 7 d in serum-free DMEM/F-12 (1 : 1) medium containing 2% B27 supplement, 20 ng/ml EGF, and 20 ng/ml bFGF. Neurospheres in 96-well plates were counted at day 1, 3, 5, and 7 according to a previously described method.\textsuperscript{10} Floating neurospheres were dissociated and plated on 6-well plates containing slides pre-coated with poly-L-lysine in serum-free medium prior to immunocytochemical analysis.

Immunocytochemistry

Cells in 6-well plates were fixed with 4% paraformaldehyde and blocked with 10% normal goat serum, then incubated first overnight at 4 °C with rabbit anti-DJ-1 polyclonal antibody (1 : 600; with TRITC-conjugated goat anti-rabbit immunoglobulin G (IgG) 1 : 200) before immunostaining for a marker of progenitors, nestin (1 : 200; with FITC-conjugated goat anti-rabbit IgG 1 : 200).

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Finally, cells were counterstained with Hoechst 33342 (1:100) and mounted with medium containing PBS (pH 8.0) and glycerol (1:9). Cells incubated in the absence of both primary antibodies were used as negative control. Fluorescence microscopy images were obtained with a confocal imaging system (Leica, U.S.A.).

**Flow Cytometry** Neurosphere suspensions were collected by centrifuging at 1200 rpm for 5 min. Cells were trituted and filtered through 70 μm nylon mesh to reach a final concentration of 1×10⁶ cells/ml. They were fixed with 2% formaldehyde, incubated with the primary antibody (DJ-1, 1:600) and then with the secondary antibody (FITC-conjugated goat anti-rabbit IgG, 1:200). Omission of the primary antibody was used as negative control. Cells were resuspended in 500 μl of buffer (2% FBS, 0.1% sodium azide in PBS) and analyzed on a FACSCalibur flow cytometer (BD Biosciences, U.S.A.).

**Immunohistochemistry** The embryo brains from E14 rats were removed and immersed in 10% paraformaldehyde for 24 h, and then in 30% sucrose for 3 d at 4 °C. They were then embedded in optimum cutting temperature (OCT) compound (Sakura, Tokyo, Japan), cut with a freezing microtome into 10 μm thick sections and then fixed in acetone. For DJ-1 and nestin immunofluorescence staining, the sections were incubated overnight at 4 °C with the primary antibodies (anti-DJ-1, 1:200; anti-nestin, 1:200), and incubated with the appropriate secondary antibodies (goat anti-rabbit IgG conjugated with FITC or TRITC, 1:200). Images were obtained with a confocal imaging system (Leica, U.S.A.).

**Statistical Analysis** Data are presented as mean±S.D. Statistical analyses were performed using an analysis of variance (ANOVA), followed by the Student’s t-test. Values of p<0.05 were considered to be significant.

**RESULTS**

**Proliferation of the Embryonic Cortical NSCs from E14 Rats** We isolated embryonic cortical neural stem cells from E14 rats. The cells were cultured in serum-free medium with cytokines bFGF and EGF. We did not use FBS throughout our stem cell culture procedure in order to avoid its various effects on stem cell cultures and to simplify the requirements of our culture. After 3 d in culture, neurospheres had formed and were suspended in the medium (Fig. 1A). The vast majority of the neurosphere cells were nestin-positive (Fig. 2). The cells were expanded in serum-free medium for

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**Fig. 1. Neurosphere Formation in the Cortex of the Fetal Rat**
(A) Neurosphere formation from embryonic cortical NSCs, phase contrast. Scale bar: 100 μm. (B) Isolated NSCs were cultured on 96-well plates, 5×10⁵ cells/cm², for 7 d. The graph shows the number of spheres per well from the fetal cortex. Experiments were independently repeated three times.

**Fig. 2. Co-expression of Nestin and DJ-1 in Proliferative NSCs** Neurospheres derived from fetal rat cortex were stained for DJ-1, nestin, and Hoechst 33342 (to label nuclei) at 1 d (A), 3 d (B), 5 d (C), and 7 d (D) during cell proliferation. A merged image is shown in the lower right panel. Scale bar for all: 20 μm.

**Fig. 3. Expression of DJ-1 in NSCs**
(A) Neurospheres from day 3 (peak 3) and day 5 (peak 2) were stained with a DJ-1 polyclonal antibody labeled with FITC; a negative control was included in each experiment (peak 1). (B) The expression of DJ-1 in NSCs on day 5 was slightly decreased relative to day 3, but this did not reach significance (p>0.05). Experiments were independently repeated three times.
7 d, and the number of neurospheres increased daily (Fig. 1B). When kept cultured in serum-free medium with bFGF and EGF, NSCs continued to proliferate for several weeks.

The Co-expression of DJ-1 and Nestin in Proliferative Embryonic Cortical NSCs

In order to observe the expression of DJ-1 in proliferating NSCs, floating neurospheres from day 1, day 3, day 5 and day 7 during proliferation were dissociated and plated on 6-well plates in serum-free medium. Neurospheres from different days were immunostained with DJ-1, nestin, and Hoechst 33342 (Figs. 2A—D). During proliferation, neurospheres had formed and were positively stained for nestin, the specific marker generally used for identifying NSCs. In the meantime, DJ-1 expression was evident in both the cytosol (Figs. 2A—D) and nucleus (Figs. 2C, D) of these cells, which is consistent with previous studies on the localization of DJ-1. These results indicated that DJ-1 and nestin are co-expressed in NSCs during proliferation.

The Expression of DJ-1 in Proliferative Embryonic Cortical NSCs

We further tested changes in DJ-1 expression in proliferative NSCs using flow cytometry. Neurospheres from day 3 and day 5 during proliferation were immunostained with an anti-DJ-1 antibody and the resulting changes were observed using flow cytometry. The mean fluorescence intensities of day 3 and day 5 neurospheres were 95.6±30.1 and 64.7±10.8, respectively (n=3). DJ-1 expression decreased slightly on day 5 relative to day 3, but there were no significant changes (p<0.05) (Figs. 3A, B).

The Expression of DJ-1 and Nestin in the Cortex of Fetal Rats

To confirm the above findings, DJ-1 and nestin
expression in the cortices of E14 fetal rats were evaluated via immunohistochemistry. Frozen sections of the cortex were immunostained with DJ-1- and nestin-specific antibodies, respectively (Figs. 4A, B). The results showed that cells in the same region of the cortex were positively stained for both nestin and DJ-1, suggesting that DJ-1 expression could be detected in cortical NSCs.

DISCUSSION

The neuropathological hallmarks of PD include progressive and profound loss of dopaminergic neurons in the substantia nigra pars compacta. Scientists currently believe that both environmental and genetic factors can cause PD. Epidemiological studies have revealed that 10% of PD cases have a strict familial etiology, whereas the majority of cases appear to be sporadic. Many genes linked to rare familial PD have been discovered during the last decade. DJ-1 was first identified and reported by Japanese scientists in 1997. Mutations and deletions in the PARK7 gene that encodes DJ-1 account for less than 1% of all familial cases of PD. Previous studies indicate that over-expression of wild-type DJ-1, either in cell culture or in dopaminergic neurons in vivo, protects against a wide variety of toxic injuries due to oxidative stress. DJ-1 also functions as a redox-dependent chaperone to inhibit α-synuclein aggregation and subsequent cell death. Furthermore, DJ-1 associates with parkin during oxidative stress. Taken together, the above evidence suggests that DJ-1 plays a crucial role in the maintenance and survival of dopaminergic neurons.

Recent studies have shown that neurogenesis occurs even in the adult central nervous system. Further investigation into the roles of DJ-1 in the proliferation and differentiation of adult NSCs may provide useful information regarding the pathogenesis of PD. Previous research has demonstrated that embryonic stem cells deficient in DJ-1 display an increased sensitivity to oxidative stress and proteasomal inhibition. In the present report, we explored the expression of DJ-1 in proliferating NSCs. The neurospheres were immunostained with nestin and DJ-1 antibodies at various stages of NSC proliferation. We found that DJ-1 was consistently co-expressed with nestin and DJ-1 antibodies at various stages of NSC proliferation. Although the expression of DJ-1 did not change during proliferation, its expression may be altered following differentiation. Therefore, further studies are required to investigate the expression of DJ-1 following differentiation of NSCs, particularly in cells that differentiate into a dopaminergic neuron phenotype.

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