Inducing dopaminergic differentiation of expanded rat mesencephalic neural stem cells by ascorbic acid *in vitro* *

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Abstract Ascorbic acid (AA) induced differentiation of neural stem cells (NSCs) into dopaminergic (DAergic) neurons is reported. NSCs derived from rat mesencephalon were maintained and expanded in a defined medium containing mitogens of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). Compared with the control, ascorbic acid treatment led to more DAergic neuronal differentiation as indicated by the expression of tyrosine hydroxylase (TH) and dopamine transporter (DAT), which are specific markers of dopamine neurons. AA induction also enhanced expression of Nurr1 and Shh. PD98059, an inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway, could block AA-induced Nurr1, TH and DAT mRNA expression. The results might suggest a new strategy to provide enough dopaminergic cells for the therapy of Parkinson's disease (PD), and Nurr1 and ERK signaling pathway might participate in the AA-induced DAergic differentiation.

Keywords: neural stem cells, differentiation, Parkinson's disease, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, signal transduction.

Recent progresses in stem cell biology and recognition of the unique biological properties of stem cells have made it possible to treat the neurodegenerative diseases including Parkinson's disease (PD) by means of cell replacement and nutritional support with neural stem cells (NSCs). Tissue reconstruction based on stem cells provides us with opportunities and some unpredicted applications. The urgent task of today is to interpret the signals modulating the expansion and differentiation of stem cells and the underlying mechanisms, to control stem cells differentiation into special defined phenotypes such as DAergic neurons, and to enhance the survival and integration of cells after transplantation.

The applausive strategy to treat PD is cell replacement[1], which requires enough functional DAergic neurons. NSCs can be used as an alternative source of transplantation to replenish the DAergic neurons. However, reliable means of inducing NSCs to generate DAergic neurons has been far from satisfactory. To find out effective inductors which could provide unlimited cell resource for transplantation might make a breakthrough in stem cell application. Previous researches have found that several factors could affect this process[2]. Recently, presence of ascorbic acid (AA) in the central nervous system (CNS) aroused great interests[3]. Brain has the highest AA level among all tissues because of the presence of a specific AA transporter system (a recently identified stereo-specific transporter) in neurons, which is responsible for keeping the steep intra/extra-cellular AA concentration gradient. AA concentration is especially high in forebrain including neostriatum which is rich in dopaminergic terminals. And substrantia nigro is the critical site controlling AA release from the neostriatum[4]. Taking these into consideration, we propose that AA may take part in the DAergic neurogenesis. On the base of long-term proliferation and expansion of mesencephalic stem cells, our research focused on the effect of AA on inducing DAergic differentiation and possibly related gene expression in this process. Using the MAPK cascade inhibitor PD98059, the signaling pathways involved were also studied.

1 Materials and methods

1.1 Materials

Wistar rats were provided by the Experimental

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Animal Center of Academy of Military Medical Science. Culture medium and growth factor (DMEM/F12, B27, EGF, bFGF) were obtained from Gibco. The antibodies against nestin, dopamine transportor (DAT), glial fibrillary acidic protein (GFAP), neuron specific enolase (NSE) and galactocerebroside C (Gal C) were purchased from Chemicon. Rat tyrosine hydroxylase (TH) antibody and ascorbic acid were the products of Sigma. The kits for RNA extraction and reverse transcription were bought from Promega.

1.2 Isolation and expansion of NSCs

NSCs were isolated from 12.5-day-old rat's mesencephalon. The tissue was microdissected and incubated in 0.125% trypsin for 30 min to obtain a single cell suspension, which were then resuspended in serum-free expansion medium DMEM/F12 supplemented with 2%B27 (Gibco), 20 ng/mL human recombinant FGF-2 (Gibco), 20 ng/mL human recombinant EGF (Gibco), 100 units/mL penicillin and 100 mg/mL streptomycin. Cells were plated at an initial concentration of 4×10^5 cells/mL in 25 cm^2 cell culture flasks and allowed to grow as microspheres. Primary culture medium was changed every 3 ~ 4 days. For passage, neurospheres were dissociated to single cells by trypsin incubation and resuspended in culture medium. Nestin expression and the ability to differentiate into neurons and glial cells of the expanded NSCs were examined.

1.3 Ascorbic acid treatment

The newly passaged cells were plated onto Poly-L-lysine pre-coated glass coverslips at a density of 5×10^4 cells/mL in 24 well plates (Costar). After 3 days culturing, the expansion medium was replaced by differentiation medium DMEM/F12 containing 2%B27, and supplemented with AA at different concentrations (1 μmol/L, 10 μmol/L, 50 μmol/L, 100 μmol/L, 200 μmol/L). The medium was half changed on the 3rd day, and then on the 6th day cells were analyzed for a number of markers specific for DAergic neuron, such as TH and DAT, using immunocytochemistry.

1.4 Gene expression during differentiation

To examine gene expression profile, cells were inoculated as above in 10 cm plates and separated into 3 groups. As the control, cells were cultured in serum-free expansion medium (DMEM/F12, 2% B27, EGF, bFGF). In the second group, cells were cultured in DMEM/F12, 2% B27, supplemented with 5%FCS. The third group was treated with AA (DMEM/F12, B27, 100 μmol/L AA). The medium was half changed on the 3rd day, and then cells were collected on the 6th day for analyzing expression of several possibly related genes, such as *TH*, *DAT*, *Shh*, *PTX3*, *Nurr1* using RT-PCR technique.

1.5 Effect of PD98059 on differentiation

PD98059 (2′-amino-3′-methoxyflavone, Calbiochem), a strong inhibitor of MEK (ERK kinase), was used to analyze whether the ERK signaling pathway was involved in the AA-induced DAergic differentiation. PD98059 was dissolved in ethanol in dark to yield 25 mmol/L stock solution. This stock solution was added into 100 μmol/L AA-induction medium in a final concentration of 25 μmol/L. The medium was half changed on the 3rd day and the cells were collected on the 6th day for analysis.

1.6 Immunocytochemistry

Cells were fixed with 4% paraformaldehyde. Nonspecific binding sites were blocked with 5% goat serum in phosphate buffered saline (PBS), and the cells were permeabilized with 0.1% Triton X-100 in PBS. Primary antibodies were incubated with the cells for 2 h at 37 ℃ and kept overnight at 4 ℃ at dilutions from 1∶500 to 1∶1000. The following primary antibodies were used: Monoclonal anti-TH (1∶500), monoclonal anti-DAT (1∶1000), monoclonal anti-nestin (1∶200), anti-GFAP (1∶500), anti-NSE (1∶200) and Gal C (1∶1000) antibodies. After being washed three times for 5 min with PBS, biotinylated secondary antibody was applied and incubated for 30 min, and after washed as above, streptavidin-conjugated HRP was added and incubated for another 30 min. DAB dying was operated as described everywhere. Then the slides were washed and stored for observing. For nestin expression, fluorescein linked secondary antibody were added to incubate with first antibody for 30 min and observed by a fluorescence microscope.

1.7 RT-PCR analysis

For RT-PCR analysis, cells were collected and subjected to TRIZOL lysis and total cellular RNA extraction was carried out according to the manufacturer's recommendation. MMLV kit (Promega) was used for reverse transcription. The following primers were used to amplify target cDNA (Table 1). PCR

was performed for 32 cycles of 94 ℃ for 30 s, 52 ℃ (TH)/55 ℃ (DAT, Nurr1, Shh, PTX3) for 30 s, and 72 ℃ for 30 s with a GENEAMP PCR System 2400 (Perkin-Elmer).

Table 1. Primers designed to detect target cDNA

cDNA	Up	Down	Product size(bp)
TH	5′-CAG AGT CTC ATC GAG GAT GC-3′	5′-CTT GTC CTC TCT GGC ACT GC-3′	376
DAT	5′-TGC TGG TCA TTG TTC TGC TC-3′	5′-ATC CAC ACA GAT GCC TCA CA-3′	202
Nurr1	5′-CTT GTA CCA AAT GCC CCT G-3′	5′-CAT CGG GCT ATG CTG TAC C-3′	196
Shh	5′-AAA AGC TGA CCC CTT TAG CC-3′	5′-TGC ACC TCT GAG TCA TCA GC-3′	198
PTX3	5′-GAG CAC AGT GAC TCG GAG AA-3′	5′-CTC AGT GAG GTT GGT CCA CA-3′	201

2 Results

2.1 NSCs expansion *in vitro*

Many cells died in the special serum-free culture medium in primary culture, while a few other cells divided rapidly and formed small clusters in a few days. Thereafter, the clusters grew larger steadily and formed proliferating spheres. After 7 days expansion, many float spheres composed of hundreds of cells could be seen. The spheres exhibited regular shape without neurite extension (Plate I, left). The medium was changed every 3 days and the cells were passaged every 6 ~ 7 days. Primary clone forming rate was very low, while increased for the following passage. These cells could be passaged continuously *in vitro*, and showed a constant expansion rate in every passage. After 15 passages, the total cell number increased by about 2.4×10^4 folds.

While proliferating, the spheres were immunoreactive for nestin, an intermediate filament found only in progenitor cells (Plate I, right). When the mitogens were withdrawn and FBS added, the cells attached to the bottom of the flasks gradually and began to show different appearances. Neuron-like cells exhibited round or ellipsoidal cell body with few long extensions; glial-like cells were thin and flat with several extensions (Plate II).

2.2 Effect of AA on NSCs differentiation

Removal of mitogens initiated random differentiation of NSCs, but the amount of TH^+ cells was rave. In contrast, exposure of the stem cells to AA increased the proportion of TH^+ cells in differentiated cell population. 100 μmol/L AA treatment led to more than 20-fold increase, and 10 μmol/L AA led to nearly 5-fold increase in TH^+ cells compared with the control (Plate III, Fig. 1). DAT protein expression also increased (Plate IV, Fig. 1). But 1 μmol/L AA treatment showed no significant function. Additionally, when AA exceeded 100 μmol/L, the ratio of TH^+ cells would not increase any more.

TH and DAT mRNA expression levels during AA induced differentiation were tested by RT-PCR. In agreement with the immunocytochemical results, AA treatment increased the expression level of TH and DAT mRNA (Fig. 1).

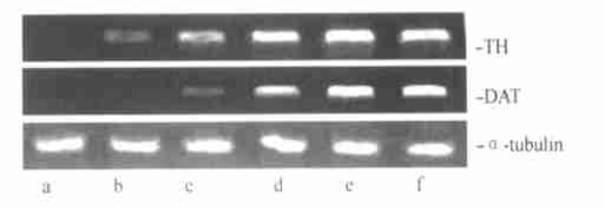

Fig. 1. TH and DAT mRNA expression induced by AA. a, Control; b, 1 μmol/L AA; c, 10 μmol/L AA; d, 50 μmol/L AA; e, 100 μmol/L AA; f, 200 μmol/L AA.

2.3 Gene expression in differentiation of NSCs

To understand the mechanisms involved in the AA-induced DAergic neuronal differentiation, we examined a number of gene expression that have been previously shown to be important for DAergic neurons generation. NSCs from mesencephalon expressed PTX3 and moderate Nurr1 but no TH, DAT or Shh. Neither FCS nor AA treatment affected expression of PTX3. 5% FCS enhanced expression of Nurr1 and DAT but not TH or Shh. 100 mmol/L AA exposing led to a high expression of TH and Shh, and even higher expression of Nurr1 and DAT compared with FCS treatment (Fig. 2).

2.4 Effect of PD98059 treatment

In order to elucidate the signal transduction pathways that might be responsible for AA induced increase in DAergic neurons, we utilized PD98059, a specific inhibitor of MAPK pathway, to examine its function. Addition of PD98059 led to death of a lot of cells. RT-PCR results showed that TH and DAT mRNA expressions induced by AA were completely inhibited; the level of Nurr1 expression decreased while Shh expression did not changed much (Fig. 2).

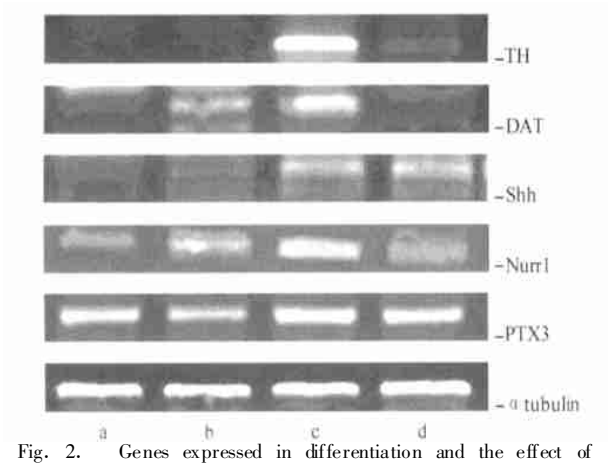

Fig. 2. Genes expressed in differentiation and the effect of PD98059 treatment. a, Control; b, FCS group; c, AA group; d, AA+PD98059 group.

3 Discussion

Cell transplantation has become a prospective therapeutic strategy with a great potential to treat human neural degenerative disorders, in which the neural cells undergo degeneration and death[5]. The recent breakthrough in stem cell research has brought about an alternative graft to treat neurological diseases by means of cell-replacement. NSCs[6] are multipotential cells that can both *in vivo* and *in vitro* generate neuron, astrocyte, and oligodendrocyte, the three main cell types in central nervous system. Stem cell transplantation might help us to overcome the intrinsic inability of regeneration of nervous tissue to replace the lost elements, and the grafted cells might produce some beneficial effects by providing host cells with additional trophic support. Because of the blood-brain barrier, there is almost no rejection response between different individuals or even different species. Furthermore, stem cells can be used as vectors for interesting gene expression by inserting the gene of functional protein into the genome of the cells. Given their capacity of self-renewal and differentiating efficiently into the desired cell types, NSCs are ideal seed cells for cell-replacement in treatment of neural diseases.

Successful cell transplantation must include survival, acquisition of special phenotypic traits and functional integration into the recipient brain circuity[7]. Research on regulating NSCs to differentiate into special cell phenotypes and the underlying mechanisms is of significance for future applications. Results of numerous experiments *in vivo* have made it clear that transplanted neural progenitor cells receive the modulation of host microenvironment signals, but the survival and differentiation to functional neurons from NSCs were very few, which suggest that only signals from the niche are far from enough[8]. Modulating the host microenvironment might be one strategy, while exploring the inducing signals that might switch NSCs differentiation before transplantation might be more effective by improving their survival and differentiation[9]. It is important to study the factors and mechanisms involved in promoting NSCs to differentiate into desired phenotype for practical use.

After 3 times passaging, the primary neurons were almost lost, so we can eliminate the primary mesencephalic DAergic TH^+ cells and examine the exclusive effect of ascorbic acid on differentiation. TH is a restriction enzyme of dopamine metabolism and a specific marker for DAergic neurons. In PD patients, TH expression is depressed. AA treatment enhanced the proportion of NSCs converting into TH^+ cells at the concentration of 10 μmol/L, and peaked at 100 μmol/L. Accordingly, AA increased the TH mRNA expression. Above 100 μmol/L, positive ratio of TH^+ cells did not change any more even the AA concentration increased, which suggested that the effect of AA had saturated. AA exposing not only increased expression of TH but also had effect on neurotransmitter synthesis and release. DAT is exclusively expressed in DAergic cells, and the protein and mRNA levels of DAT also increased through AA inducing. Our research has exhibited the possibility of AA in promoting NSCs to differentiate into DAergic neurons.

NSCs from mesencephalon expressed PTX 3 and weekly expressed Nurr1 but not TH, DAT or Shh. AA induction did not affect expression of PTX3 but enhanced expression of Nurr1, Shh, TH and DAT. PTX3, a homeodomain-containing transcription factor, was expressed from day 11 on DAergic neuronal precursors, and they are the only cell population expressing PTX3 in the CNS. Its expression is maintained throughout the adult life in human and mouse. It was suspected that PTX3 activates tyrosine hydroxylase promoter through a high-affinity binding site, which is sufficient for PTX 3 responsiveness[10]. As a site special gene, PTX3 did not show obvious changes after FCS and AA treatment, which suggests that PTX3 might not be involved in NSCs differentiation. Nurr1 belongs to the orphan nuclear receptor superfamily and is predominantly expressed in central DAergic system and plays an important role in devel-

opment and survival of mesencephalic DAergic neurons[11]. Embryonic stem cells overexpressing Nurr1 can differentiate into dopamine neurons *in vitro* and in PD rat model. Consistent with expression of TH and DAT, expression of Nurr1 was enhanced after AA induction. This suggests that Nurr1 may take part in the AA-induced differentiation. When FCS was used as an inducer, there were more glial cells and fewer DAergic cells compared with AA induction. This can interpret why Nurr1 was intensively expressed in AA group than in FCS group. Shh is regarded as the modulator in NSCs differentiating towards neurons, the enhanced expression of which after induction matches the case that NSCs differentiation was activated. And different ratio of neurons and glial cells can explain its different expression level in AA group and FCS group.

The MAPK cascade plays a crucial role in transducing of a wide range of extracellular stimulates into responses governing growth and differentiation[12]. Among MAPK superfamily, ERK plays a key role in cell proliferation, cell cycle arrest, and terminal differentiation, whereas JNK/SAPK and p38 kinase are thought to inhibit cell growth and induce apoptosis. To assess the role of ERK in the AA-induced differentiation process, we inhibited ERK pathways by PD98059. PD98059 is a selective and cell-permeable inhibitor of MEK (ERK kinase) that does not inhibit SAPK/JNK or p38 kinase pathways[13]. In our study, a lot of cells died when PD989059 was added into AA-inducing system, confirming that MAPK cascade is critical for cell survival.

The finding that AA-induced expressions of TH and DAT were totally blocked by PD98059 suggested that ERK signal pathway was involved in AA-induced DAergic differentiation. Decreased Nurr1 expression indicated that Nurr1 at least plays a role in AA induction. The details of the signal transduction and the exact role of this pathway remain to be elucidated. Whether there are other signal pathways involved needs further research. The finding that Shh expression did not change much after PD98059 treatment implies that ERK might not be in the Shh pathway or not a target of Shh.

Antioxidant agents including ascorbic acid have shown to improve survival of mesencephalic DAergic neurons in primary cell culture and to counteract the toxic effects *in vitro* or *in vivo*[14]. However, the molecular mechanisms regulating NSCs differentiation are not well understood yet. Whether the mechanisms of AA's effect on DAergic differentiation are related to its antioxidant function need further research. But Yan et al.[15] found that effect of AA on differentiation cannot be mimicked by other antioxidants. AA's effect of successful conversion of fetal progenitor cells *in vitro* to the desired neuronal phenotype provides a new strategy for a large scale generation of DAergic neurons for PD treatment. The research also suggests that Nurr1 takes part in AA-induced DAergic differentiation. AA may promote NSCs differentiating into DAergic neurons through ERK signal pathway. But the exact role of this pathway remains unknown. Studies on inducing NSCs to differentiate into desired phenotype, seeking for highly effective inducers and identifying the molecular mechanisms underlying which would provide us with a solution to the problems encountered in treating the neural degenerative diseases. Researches in these areas are just on the way.

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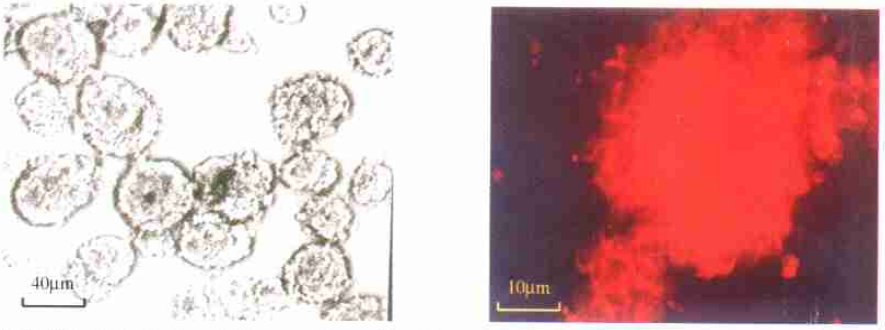
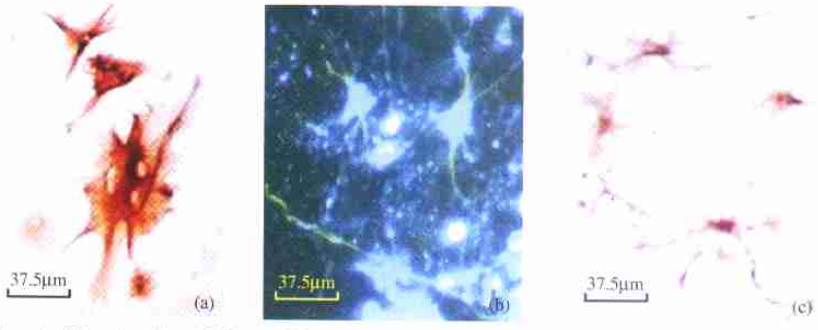
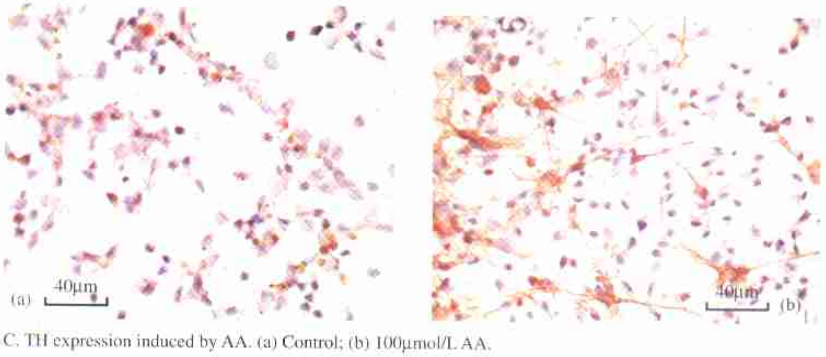
Plate I



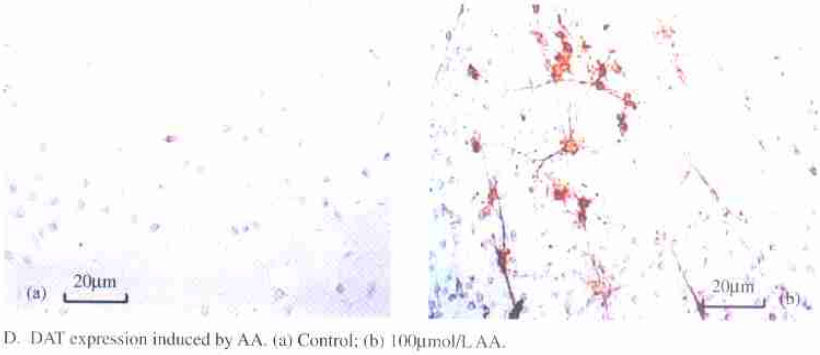
A. Proliferating neurospheres in the serum-free medium (left), and nestin expression of the neurospheres (right).



B. The cells differentiated from NSCs. (a) GFAP $^+$, (b) NSE $^+$, (c) Gal C $^+$.



C. TH expression induced by AA. (a) Control; (b) 100μmol/L AA.



D. DAT expression induced by AA. (a) Control; (b) 100μmol/L AA.