Original Article
Bone marrow stromal stem cells induce autophagy that may exert neuroprotective effects

Weiling Dong¹*, Qi Fang¹*, Hong Gao², Ju Zhou², Jing Wang¹, Dapeng Wang¹, Liqiang Yu¹, Guozhen Hui¹, Lidong Shan²

¹Department of Neurology, First Affiliated Hospital of Soochow University, 188 Shizi Street, Suzhou 215006, China; ²Department of Physiology and Neurobiology, Medical College of Soochow University, Suzhou 215123, China. *Equal contributors.

Received August 5, 2016; Accepted February 24, 2017; Epub May 15, 2017; Published May 30, 2017

Abstract: Purpose: Bone marrow stromal cells (BMSCs) are self-renewing, multipotent cells that can migrate to pathological sites, making them a promising therapeutic tool for neurodegenerative disorders. However, the therapeutic mechanisms by which they act are largely unknown, although cell replacement and paracrine mechanisms have been hypothesized. The objective of this work was to determine the ability of rat BMSCs to induce autophagy and whether this effect provides neuroprotection. Methods: We co-cultured BMSCs with rotenone-exposed SH-SY5Y cells overexpressing α-synuclein, and examined whether BMSCs could induce autophagy. Results: The BMSC co-culture led to increased expression of autophagy markers, such as the microtubule-associated protein light chain 3 II (LC3II) and Beclin1, and decreased levels of α-synuclein in A53T mutant cells. This suggests that BMSCs can induce autophagy and that this effect may enhance the clearance of aggregated α-synuclein. In addition, BMSCs were also found to protect against rotenone-induced SH-SY5Y cell injury and apoptosis, and this effect became enhanced following rapamycin treatment and was partially decreased following treatment with the autophagosome inhibitor 3-methyladenine (3-MA). Conclusion: Ultimately, we conclude that BMSCs induce autophagy, an effect which may have neuroprotective benefits. BMSC transplantation is a potentially effective strategy to treat diseases associated with aggregated proteins, such as Parkinson’s disease (PD).

Keywords: BMSCs, autophagy, co-culture, rotenone, α-synuclein

Introduction
Parkinson’s disease (PD) is a common neurodegenerative disorder clinically characterized by bradykinesia, resting tremor, rigidity and postural instability [1]. Its pathological hallmarks include loss of the dopamine (DA) neurons in the substantia nigra and development of cytoplasmic protein inclusions (known as Lewy bodies) in the surviving DA neurons. Although great progress has been made throughout the past two decades toward understanding PD pathogenesis, its exact etiology and pathogenesis remains unclear. However, the collective body of research has indicated that interactions between environmental factors and genetic susceptibilities likely contribute to PD pathogenesis [2].

Despite these findings, most existing PD models are induced by a single factor, such as neuro-pathological insult. Thus, to develop a more reliable PD model, we combined environmental (rotenone) and genetic factors (gene mutation) in our study. Rotenone is a commonly used pesticide in China that inhibits mitochondrial complex I activity, initiates reactive oxygen species production, and accelerates α-synuclein fibril formation. In fact, the first PD-associated gene discovered was α-synuclein (SNCA), and since then three different missense mutations in SNCA (A30P, A53T, and E46K) have been identified as risk factors for early-onset, autosomal dominant PD. These genetic alterations induce α-synuclein aggregates [3]. Several studies have shown that expression of an α-synuclein A53T mutant in SH-SY5Y cells induces α-synuclein aggregates and faster fibril formation, as compared to WT α-synuclein or the A30P mutant [4]. Therefore, we chose cells expressing the A53T mutant to explore whether BMSCs enhance α-synuclein degradation.
PD is an incurable multifactorial disease, and the most efficient therapeutic treatments currently available are targeted at symptom improvement, including drug therapy, surgical interventions and stimulation therapy. However, these strategies only ameliorate early stage clinical symptoms and do not stop or reverse DA neuron degeneration. In recent years, cellular transplantation has shown promise as a new therapy. Bone marrow stromal cells (BMSCs) have many advantages compared to fetal cells or embryonic stem cells. One advantage is that BMSCs can be easily cultured and expanded, and they are safer than stem cells derived from other sources because of their low tumorigenic potential. Furthermore, few ethical issues and low immune rejection make BMSCs ideal candidates for PD therapy.

Previous animal studies have shown that BMSC transplantation induces behavioral recovery and increases DA in the substantia nigra pars compacta and striatum [5]. Recent clinical studies have also shown that autologous adult human (h) BMSC transplantation to PD patients induced clarity in speech, a reduction in tremors, rigidity, and freezing attacks [6]. However, the mechanisms by which BMSCs promote neurological recovery are largely unknown. Several studies have shown that BMSCs can transdifferentiate into neuronal-like cells or glial cells, like neural progenitor cells or T helper immunoreactive cells [7].

Growing evidence has suggested that BMSCs secrete many neurotrophic factors, including brain-derived neurotrophic factor, which stimulate endogenous neural stem cell migration and differentiation [8], leading to endogenous repair and functional improvement. However, limited published data have shown whether the neuroprotective effects of BMSCs are related to autophagy enhancement. Our previous study (unpublished data) found that incubation with BMSC-derived conditioned media significantly increased the levels of microtubule-associated protein light chain 3 II (LC3 II), an autophagosome marker. These findings raised the question of whether BMSCs protect DA neurons by increasing autophagosomes.

In this study, we used cell co-cultures and the autophagy inducer rapamycin or the autophagy inhibitor 3-methyladenine (3-MA) to explore whether BMSCs protect against rotenone-induced cell toxicity in α-synuclein-overexpressing SH-SY5Y cells and to determine whether this event may be related to autophagy.

**Materials and methods**

**Establishment of α-synuclein-overexpressing SH-SY5Y cells**

SH-SY5Y neuroblastoma cells are often used as a PD cell model because they express dopamine-β-hydroxylase. The SH-SY5Y cells were cultured in DMEM medium (Gibco, Carlsbad, California, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, Utah, USA) at 37°C in a humidified 5% CO₂ atmosphere. To generate SH-SY5Y cells overexpressing wild-type (WT) α-synuclein, RNA was extracted from the chronic myeloid leukemia-derived K562 cell line obtained from Dr. Zhao (Cyrus Tang Hematology Center, Soochow University), as α-synuclein is highly expressed in peripheral blood mononuclear cells, plasma and cerebrospinal fluid. RNA was reverse-transcribed into cDNA with reverse transcriptase by use of the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Basel, Switzerland), and human SNCA was amplified by RT-PCR. The primer sequences were as follows: P1, GGAAGATCTGTGTGGTGTAAAGGAATT-CATT; P2, GGAAGATCTAGAAACTGGGAGCAAAG-ATA. The familial PD-linked A53T mutation was generated by site-directed gene mutagenesis using a primer containing the mutated nucleotide and a site-directed gene mutagenesis kit (Beyotime, Jiangsu, China).

The orientation and sequence of each construct was confirmed by restriction enzyme analysis and sequencing. The gene was ligated into the eukaryotic expression vector pLentiVENUS-YFP (kind gift from Dr. Zhao, Cyrus Tang Hematology Center, Soochow University). The pLentiVENUS-YFP-SNCA lentivirus was generated in 293T cells by the calcium phosphate precipitation method (http://tronolab.epfl.ch). The lentivirus was then infected into SH-SY5Y cells (gift from Dr. Liu, Institute of Neuroscience, Soochow University). YFP-positive cells were sorted and collected by flow cytometry (BD FACSAriaIII, Franklin Lakes, New Jersey, USA). Finally, α-synuclein expression levels were assessed by inverted fluorescence microscopy (Axio Scope A1, Goettingen, Germany), RT-PCR and immunoblotting.
BMSC isolation

BMSCs were extracted as previously described from adult male Sprague-Dawley rats, weighing 200-250 g, that had been purchased from the Shanghai SLAC Laboratory Animal Co. Ltd. [5, 9]. The rats were housed at room temperature (temperature 20-24°C; humidity 55±10%; 12 h light/dark cycle with lights on at 7:00 AM) with food and water available ad libitum. Animals were anesthetized with chloral hydrate (100 mg/kg body weight in 0.9% NaCl), and the femurs and tibias were carefully dissected away from the attached tissue.

The marrow was removed with sterile phosphate-buffered saline (PBS), after which the mononuclear cell layer was isolated by Ficoll-Hypaque solution, washed in PBS and cultured at a density of 10×10^5 cells/cm^2 in DMEM (Gibco) supplemented with 10% FBS (Hyclone) at 37°C in 5% CO_2 and 95% air. After 48 h of incubation, the non-adherent cell population was removed by replacing the medium. Upon reaching 80% confluence, the cells were resuspended in 0.25% Trypsin (Sigma, Carlsbad, California, USA) without EDTA and passaged. Finally, the third passage of BMSCs was used for experimental analysis.

Immunophenotyping of BMSCs was performed using flow cytometry to identify the presence of specific cell-surface antigens. Cultured BMSCs were collected in PBS and blocked in 5% BSA for 2 h. Cells were then incubated with FITC- or phycoerythrin-conjugated antibodies against CD44, CD45, CD34, or CD29 (1:500) (BD Pharmigen, San Diego, California, USA) for 30 min. Data were acquired from 10,000 cells using a FACSCalibur analyzer and Cell Quest-Pro software (BD Biosciences, Franklin Lakes, New Jersey, USA).

This study was evaluated and approved by the Ethical Committee of Soochow University and performed according to the guidelines of the National Institutes of Health for the care and use of laboratory animals (NIH Publication No. 80-23).

Cell co-culture and drug treatment

A 20 mM stock solution of rotenone (Sigma) was prepared in dimethyl sulfoxide (DMSO) and diluted in serum-free medium prior to addition to cultures at the desired final concentrations. The specific autophagosome inhibitor 3-MA (Sigma) was prepared as a 100 mM stock by heating in PBS, and was added to the cultures at a final concentration of 5 mM. Rapamycin (Selleck Chemicals, Houston, Texas, USA) was dissolved in DMSO at concentration of 1.0 µM before being diluted in culture medium to a final concentration of 0.2 µM. Cells were pretreated with rapamycin and 3-MA for 24 h, followed by rotenone addition. Vehicle alone was used as a control for each drug condition.

Transwells (Corning, Corning, New York, USA) were used to study the effect of BMSCs on SH-SY5Y cells overexpressing A53T α-synuclein, with BMSCs in the upper chamber and SH-SY5Y cells in the bottom chamber. The co-culture system does not enable any direct cell-to-cell contact between SH-SY5Y cells and BMSCs, hence intercellular communication only occurs through substances released by either cell type into the shared culture medium. The cell cultures were incubated at 5% CO_2, 37°C for 4 h. The upper chambers containing BMSCs were then removed, and the SH-SY5Y cells in the bottom chambers were used for subsequent analyses.

MTT assay

Cell viability was measured by MTT assay as described previously [10]. Cells were cultured in 96-well plates at a density of 2×10^3 cells per well. After each treatment, 10 µl of 5 mg/mL MTT solution was added to each well and incubated for 3 h at 37°C to allow purple formazan crystal formation. A total of 100 µL DMSO was added to each well to dissolve the formazan crystals. Finally, the optical density was measured at 570 nm.

Apoptosis analysis

Apoptosis was analyzed using the Annexin V-PE apoptosis detection kit (BD Biosciences) and flow cytometry (BD Biosciences) according to the manufacturer’s protocol. After different treatments, 1×10^6 cells/mL SH-SY5Y cells overexpressing A53T mutant α-synuclein were treated with 0.25% trypsin, washed in PBS and resuspended in 1× Binding Buffer. Cells were subsequently incubated in PE-conjugated Annexin V and 7-AAD at 37°C for 15 min in the dark. Specific fluorescence was detected using a FACSCalibur (BD) analyzer within 1 h of label-
Neuroprotective effect of BMSCs on PD

The percentage of apoptotic cells was quantified as the percentage of Annexin-V-PE-positive and 7-AAD-negative and Annexin-V-PE-positive and 7-AAD-positive cells.

**Immunoblotting analysis**

After each treatment, cells were washed three times in cold PBS and lysed in cell lysis buffer (Beyotime). After incubation on ice for 30 min, lysates were centrifuged at 12,000×g at 4-8°C for 30 min. Total protein concentration was determined by BCA protein assay kit (Beyotime). After boiling for 5 min, equal amounts of protein lysates were loaded and separated by 12% SDS-PAGE and transferred to PVDF membrane (Beyotime). Nonspecific sites were blocked in 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h. Membranes were then incubated at 4°C overnight in the following primary antibodies: anti-α-synuclein antibody (Sigma, 1:1000), anti-LC3-II antibody (Sigma, 1:1000), anti-Beclin1 antibody (Cell Signaling Technology, Boston, Massachusetts, USA, 1:1000) and anti-β-actin (Cell Signaling Technology, 1:1000) as a loading control. Membranes were then washed three times with TBS/Tween-20 and incubated in the corresponding HRP-conjugated secondary antibodies at room temperature for 1 h. After washing, membranes were subjected to ECL detection using ECL solutions (Beyotime). Relative band intensities were determined using Image Lab 3.0 software (Bio-Rad, Hercules, California, USA). All results were repeated at least three times.

**Statistical analysis**

All measurements were repeated at least three times for each experiment, and the data were expressed as mean ± SEM. All data were analyzed by t-test or one-way ANOVA followed by Tukey’s post-test using the GraphPad Prism software (San Diego, California, USA). A value of *P*<0.05 was considered significant.

**Results**

**Generation of α-synuclein-overexpressing stable cell lines**

To generate a stable SH-SY5Y cell line overexpressing A53T α-synuclein, we stably transfected cells with lentivirus expressing A53T mutant human α-synuclein or empty pLentiVENUS construct as a control. We confirmed plasmid expression by fluorescence microscopy (Figure 1A), as the plasmid contained YFP linked to an internal ribosome entry site (IRES), allowing for similar expression rates of both α-synuclein and YFP (Figure 1A) and visualization of cells expressing human α-synuclein. We confirmed α-synuclein protein expression by immunoblot...
and found that A53T α-synuclein constructs were expressed, but we did not detect α-synuclein expression in control cells transfected with vector only nor in cells that were untransfected (Figure 1B). We confirmed α-synuclein gene expression by RT-PCR. All cells expressed some α-synuclein, but those transfected with A53T mutant α-synuclein showed an increase in α-synuclein mRNA levels, as compared to cells transfected with vector only and cells that were untransfected (Figure 1C).

Rotenone induced SH-SY5Y cell autophagy

We treated A53T α-synuclein-overexpressing SH-SY5Y cells with various concentrations of rotenone (0, 2, 4, 6 or 8 μM) for various incubation times (2, 4 or 8 h) and performed Western blotting to determine whether rotenone interfered with autophagy by analyzing the expression of LC3-II and the early macroautophagy marker Beclin1 (Figure 2). A53T α-synuclein-expressing SH-SY5Y cells significantly upregulated LC3-II protein levels after rotenone treatment compared to control cells, and LC3-II levels peaked in 4 μM rotenone (P<0.01) but decreased at higher concentrations (Figure 2A-C). Thus, we used 4 μM rotenone for subsequent experiments. As shown in Figure 2D, LC3-II protein levels initially increased upon rotenone treatment, peaked at 4 h (2.42±0.26, P<0.05), and then decreased thereafter. Beclin1 exhibited similar dynamics upon rotenone treatment.

BMSCs induced autophagy in A53T α-synuclein-overexpressing SH-SY5Y cells

We treated A53T mutant cells with 4 μM rotenone for 4 h and co-cultured the SH-SY5Y cells and BMSCs in Transwells, with the BMSCs in the upper chamber. Flow cytometry analysis of BMSCs demonstrated that these cells expressed the cell-surface molecules CD29 and CD44, but were negative for CD34 and CD45 (data not shown). After 4 h rotenone treatment, we removed the BMSCs, extracted total protein from the SH-SY5Y cells, and then performed immunoblotting. The results indicated that the LC3 II/β-actin ratio was increased in BMSC co-culture (3.22±0.08) compared to control cells (2.33±0.06, P<0.05) (Figure 3D). These data indicate that BMSCs induce autophagy in A53T α-synuclein-overexpressing SH-SY5Y cells. We confirmed that proteins were equally loaded by blotting for β-actin. In addition, BMSCs decreased α-synuclein protein levels (0.93±0.06) compared to control cells (1.04±0.06) (Figure

Figure 2. Effects of rotenone on autophagy in α-synuclein-overexpressing SH-SY5Y cells. A-C. Rotenone has a dose-dependent effect on autophagy in A53T mutant α-synuclein cells after 2 h, 4 h and 8 h as determined by measuring LC3II and Beclin1 protein levels by immunoblotting. D. 4 μM rotenone treatment has a time-dependent effect on A53T mutant α-synuclein cell autophagy. The normalized-to-actin ratio of LC3II/β-actin or Beclin1/β-actin was evaluated by Image Lab 3.0 software. *P<0.05, **P<0.01 compared to controls.
Neuroprotective effect of BMSCs on PD

Increased LC3 II/β-actin ratio. These data suggest that BMSCs may reduce A53T protein accumulation, an event that may be related to enhanced autophagy. Consistent with α-synuclein proteins being regulated by autophagy, rapamycin treatment decreased their expression level (0.89±0.08) and 3-MA increased their expression level (0.99±0.10) (Figure 3B). Beclin1 expression also corresponded to changes in LC3-II levels (Figure 3C).

BMSCs protect against rotenone-induced SH-SY5Y cell injury

To further determine whether BMSCs exert neuroprotective effects, we performed MTT assays to measure cell viability. Following the
BMSCs co-culture and rotenone treatment, we removed the BMSCs, imaged the SH-SY5Y cells on an inverted microscope, and performed MTT assays to measure cell viability (Figure 4).

The BMSCs protected the SH-SY5Y cells from rotenone toxicity, as evidenced by findings from cell morphology analysis and MTT analysis (0.50±0.002) and as compared to the controls without BMSCs (0.46±0.001, P<0.05). To determine whether autophagy played a role, we pretreated the SH-SY5Y cells with the autophagy inhibitor (3-MA) or inducer (rapamycin) for 24 h. The results showed that the rapamycin pre-treatment enhanced the BMSC-mediated neuron-protective effects (0.76±0.002, P<0.01), while that of 3-MA blocked them (0.33±0.002, P<0.01), suggesting that the neuroprotective effect of BMSCs was partially due to autophagy induction.

**BMSCs protect against rotenone-induced apoptosis**

To determine whether BMSCs affected rotenone-induced apoptosis, we double stained SH-SY5Y A53T α-synuclein-overexpressing cells with Annexin-V/7-AAD. The results showed that BMSCs can decrease the apoptosis ratio of rotenone-treated A53T cells (34.42±3.85), as compared to the control without BMSCs (51.58±4.34, P<0.05) (Figure 5). Rapamycin treatment led to a more obvious decrease (30.11±4.39, P<0.05), but 3-MA increased the apoptosis rate (45.01±4.22). Our results indicated that BMSCs protected the A53T mutant SH-SY5Y cells against rotenone-induced apoptosis and suggested that this effect may be related to autophagy induction because the autophagy inducer inhibited apoptosis, while the autophagy inhibitor increased the apoptosis rate.

**Discussion**

Accumulating data suggest that BMSCs exert neuroprotective effects through cell replacement and paracrine mechanisms [5, 7]. In this study, we demonstrated that BMSCs can enhance autophagy, decrease α-synuclein aggregation, and protect α-synuclein-overexpressing SH-SY5Y cells from rotenone-induced injury and apoptosis. These neuroprotective effects of BMSCs were enhanced by rapamycin and partially blocked by 3-MA treatment. Our study suggests that BMSCs induce autophagy and that this event may exert a neuroprotective effect. Therefore, BMSC transplantation may be a useful treatment option for neurodegenerative diseases.

PD is a common neurodegenerative disease with multifactorial etiopathogenesis, including both environmental and genetic factors [2]. Therefore, we developed a more reliable PD model by combining environment factors (rote-none) and genetic factors (gene mutation). Rotenone treatment can phenocopy many of
Neuroprotective effect of BMSCs on PD

the motor symptoms and histopathological PD features in animal models, including Lewy body formation. Thus, we used rotenone as a neurotoxin in this study. To further identify rotenone’s effect on autophagy, we treated cells with various concentrations of rotenone (0, 2, 4, 6 and 8 μM) for various times (2, 4 and 8 h). We found that autophagy increased until it peaked at 4 h, after which it decreased; this finding is in line with those from a related previous study [11]. Therefore, we suggest that rotenone disrupts mitochondrial respiration, thereby increasing reactive oxygen species formation and accelerating autophagy formation [12]. However, at later time points, the autophagy-related protein would become exhausted, resulting in decreased autophagy [11]. The effect of rotenone on Beclin1 levels was consistent with its effects on LC3-II, suggesting that rotenone induces autophagy-mediated cell death through a Beclin1-dependent pathway, which is in the PI3 Kinase class III pathway.

Autophagy is a multi-step process that mediates long-lived protein and organelle degradation. The deregulation of autophagy may play a critical role in neurodegenerative disease pathogenesis, including that of PD. Several reports have recently shown that autophagy induction attenuates cellular toxicity in PD models [13, 14]. We found that incubation with BMSC-derived conditioned media significantly increased LC3 II expression (data not shown). Consistent with previous data that mesenchymal stem cells enhance the number of LC3 II-positive autophagosomes [9], our results suggest that BMSC co-culture can increase LC3 II and Beclin1 expression. This effect was enhanced by rapamycin and blocked by 3-MA treatment. Interestingly, rapamycin also increased Beclin1 expression. Rapamycin enhances autophagy by inhibiting mTOR activity, while Beclin1 induces autophagy independently of mTOR and instead interacts with Vps34 and PI3 Kinase. We propose that Beclin1 is a multifunctional protein that functions in several key cellular pathways; further studies are necessary to explore the possible mechanisms. The present data suggest that BMSCs act as autophagy inducers.

α-synuclein is the principal component of Lewy bodies, a pathological hallmark of PD. It has been previously reported that α-synuclein overexpression is toxic to neuronal cells, as it impairs the protein degradation pathway and causes subsequent neuronal cell death. Previous reports have shown that the intracellular accumulation of α-synuclein in rat brains by intranigral infusion of AAV-α-synuclein led to significant impairments in motor ability and loss of nigrostriatal cells [15]. Many in vitro α-synuclein overexpression models have also demonstrated that abnormal α-synuclein accumulation leads to mitochondrial degeneration, resulting in oxidative stress, and ultimately cell death [16]. Based on evidence that aggregated α-synuclein is cytotoxic, some studies have suggested that increasing the clearance of α-synuclein aggregation could be a therapeutic approach for treatment of PD. Previous reports have shown that increased autophagy accelerates protein aggregate clearance [17]. Here, we demonstrated that BMSCs increase the expression of the autophagy marker LC3II, with a concomitant decrease in cells overexpressing A53T mutant α-synuclein. These data suggest that BMSCs enhance the degradation of aggregated α-synuclein and that this event may be partially mediated by autophagy enhancement.

Consistent with previous reports that autophagy inhibition promotes apoptosis and stimulation of macroautophagy reduces apoptosis [18, 19], we found that co-culture with BMSCs decreased rotenone-induced apoptosis of A53T mutant SH-SY5Y cells. Rapamycin inhibited apoptosis, while 3-MA increased the rate of apoptosis. Accordingly, there are multiple connections between apoptotic and autophagic processes under some situations, and the autophagy proteins p62 and Beclin1 likely regulate apoptotic proteins.

Conclusion

Our findings demonstrate that BMSCs induce autophagy, and this event may exert neuroprotective effects, suggesting that BMSC transplantation may be an effective therapeutic approach for PD.

Acknowledgements

This work was supported by grants from the Suzhou City Foundation of China (SYS201103) and A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions. We would like to express our
Neuroprotective effect of BMSCs on PD

appreciation to Dr. Zhao (Cyrus Tang Hematology Center, Soochow University) for providing the eukaryotic expression vector pLentiVENUS-YFP and the K562 cells and to Dr. Liu (Institute of Neuroscience, Soochow University) for providing the SH-SY5Y cells.

Disclosure of conflict of interest

None.

Address correspondence to: Lidong Shan, Department of Physiology and Neurobiology, Medical College of Soochow University, Suzhou 215123, China. E-mail: danlidong@suda.edu.cn

References


