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# Exosomes of BV-2 cells induced by alpha-synuclein: Important mediator of neurodegeneration in PD

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## HIGHLIGHTS

- Alpha-synuclein can induce an increase of exosomal secretion by microglia.
- Activated exosomes expressed a high level of MHC class II molecules and mTNF-α.
- Activated exosomes can cause increased apoptosis.
- Exosomes might be important mediator of neurodegeneration in PD.

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## ABSTRACT

Parkinson's disease (PD) is a progressive neurodegenerative disease. Alpha-synuclein aggregation, which can activate microglia to enhance its dopaminergic neurotoxicity, plays a central role in the progression of PD. However the mechanism is still unclear. To investigate how alpha-synuclein affects the neuron, exosomes were derived from alpha-synuclein treated mouse microglia cell line BV-2 cells by differential centrifugation and ultracentrifugation. We found that alpha-synuclein can induce an increase of exosomal secretion by microglia. These activated exosomes expressed a high level of MHC class II molecules and membrane TNF-α. In addition, the activated exosomes cause increased apoptosis. Exosomes secreted from activated microglias might be important mediator of alpha-synuclein-induced neurodegeneration in PD.



## 1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease, the clinical manifestations of which include resting tremor, rigidity, bradykinesia, and gait disturbance [2]. The characteristic pathology of PD is degeneration of dopaminergic neurons in the substantia nigra pars compacta and the presence of Lewy bodies (LBs) [24]. LBs are spherical eosinophilic cytoplasmic protein aggregates composed of numerous proteins, which include high levels of alpha-synuclein [22]. Alpha-synuclein is an abundant 140-residue neuronal protein, which is found mainly in neuronal presynaptic terminals, close to synaptic vesicles, under physiological conditions. Studies showed that alpha-synuclein aggregation contributes to the pathogenesis of PD. However, the mechanisms remain unclear. Aberrant alpha-synuclein causes mitochondrial fragmentation and neuronal death via an effect on the mitochondrial membrane [17]. Additionally, alpha-synuclein is able to activate microglia and release pro-inflammatory cytokine and chemoattractant cytokines [8].

Microglia are the resident innate immune cells in the brain, and play a major role in injury and disease, including neurodegeneration, stroke and brain tumors. It is evident that microglia can have both neuroprotective and neurotoxic effects [8]. Over-activation and dysregulation of microglia contribute to neuronal damage in neurodegenerative diseases. Microglia become activated in the presence of aggregated and nitrated forms of alpha-synuclein and enhance alpha-synuclein-mediated neurotoxicity by increasing their number and secreting further pro-inflammatory mediators [10]. Microglia-derived inflammatory factors can regulate the progression of neuronal cell death in PD [13]. An assumed propagation path is the release and uptake of exosomes between microglia.

*Abbreviations:* PD, Parkinson's disease; LBs, Lewy bodies; CM, culture medium; FWB, flow cytometry wash buffer; APCs, antigen-presenting cells; TACE, TNF-α converting enzyme.

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Exosomes are membrane-bound vesicles of endocytic origin released by numerous cell types and are found in abundance in body fluids. Some well-characterized functions include protein secretion [4,29], immune response regulation [18,27], antigen presentation [12], RNA/protein transfer [25,28] and cell–cell interaction [5]. A recent study revealed that exosomes can transfer alpha-synuclein which might be related to the microglia-mediated progression of PD [1].

The experiments here investigated exosomes derived from mouse microglia which were induced by alpha-synuclein. We found that exosomes secreted by activated microglia was much higher than that of the control group and these exosomes expressed a high level of MHC class II and mTNF-α.

## 2. Material and methods

### 2.1. Cell culture

BV-2 microglias were cultured in RPMI1640 (Invitrogen, Long Island, NY) containing 5% fetal bovine serum at 37 °C in an atmosphere of 5% $CO_2$. The experimental cells were divided into two groups, of which without any treatment were control group, and the other treated with alpha-synuclein (Invitrogen, Long Island, NY) were study group. 0.2 μM aggregative alpha-synuclein was added to the alpha-synuclein group and co-cultured with BV-2 cells for 6 h.

### 2.2. Exosome isolation

The culture medium (CM) supernatant was collect and transferred into 30- or 50-mL polypropylene tubes. The samples were centrifuged at 300 × *g* for 10 min at 4 °C, to remove any free cells. The CM supernatant was carefully collected with a pipette (supernatant should not be poured off). The CM was transferred into a fresh centrifuge tube and spun at 2000 × *g* for 20 min at 4 °C to remove large cell particles and cell debris. Then filter the supernatant through a 0.2 μm filter to remove particles larger than 200 nm, transfer the supernatant to new ultracentrifuge tubes and seal the tubes before at 110,000 × *g* for 60 min at 4 °C to pellet the exosomes.

### 2.3. Electron microscopic analysis

Electron microscopic analysis was performed on isolated vesicle fractions as follows. Immediately after the 110,000 × *g* centrifugation step, the pellets were resuspended in 30 μL PBS (pH 7.4) containing 5 mmol/L EDTA, and were fixed by adding an equal volume of 2% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). All of the fractions from the gradient were adsorbed for 10 min onto formvar-carbon coated grids by floating the grids on 10 μL drops placed on parafilm. Grids with adhered vesicles were rinsed in PBS and examined under the electron microscope after uranyl staining and embedding. Sections were viewed using a Philips CM12 transmission electron microscope operating at 80 kV. Micrographs were prepared to a known scale.

### 2.4. Flow cytometry

Anti-CD63 coated beads was used to trap the exosomes. Incubate the exosome-bead complexes with 50 μl IgG antibody (Invitrogen, Long Island, NY) at 4 °C. Wash the exosome-bead complexes twice in wash buffer. Then add 10 μl anti-CD63 antibody (Invitrogen, Long Island, NY) to the exosome-bead complexes and incubate for 40 min under gentle movement. Wash the complexes two times in wash buffer. Add 300 μl wash buffer and analyzed in a Facs Calibur flow cytometer (Becton Dickinson, Mountain View, CA) at a wavelength of 488 nm. Fluorescence and light scatter data were obtained at logarithmic settings.

To detect surface TNF-α converting enzyme (TACE) protein expression, cells ($5 \times 10^5$ cells/0.5 mL in 24-well plates) were fixed using 1% paraformaldehyde on ice for 10 min and washed using 2% FBS flow cytometry wash buffer (FWB). The resulting cells were sequentially stained with primary anti-mouse TACE for 30 min and 2.5 μg/mL of FITC-conjugated secondary antibodies for 30 min on ice. After staining, cells were fixed using 1% paraformaldehyde on ice for 10 min and centrifuged to remove the formaldehyde, before being reconstituted with 1 mL FWB. Flow-cytometric analysis was performed as described above. The results were calculated from 10,000 acquired events.

### 2.5. Western blot analysis

Samples were separated by 10% SDS-PAGE. Proteins were transferred to PVDF membranes and blotted with mouse anti-MHC II or anti-TNF-α antibody (Invitrogen, Long Island, NY). After extensive washing, the membrane was incubated with a secondary antibody conjugated to peroxidase (KPL, Gaithersburg, Maryland). For detection of TNF-α, a recombinant soluble TNF-α was used as internal standard. Immunoreactive bands were detected by a chemiluminescent western blot detection kit (Amersham, Piscataway, NJ) according to the manufacturer's instructions.

### 2.6. Apoptosis assay

Cortical neurons from rats anesthetized with ketamine were dissected and incubated in 0.025% trypsin solution. Then cells were washed with RPMI-1640 supplemented with 5% heat inactivated FBS and re-suspended at $5 \times 10^5$ cells/mL. These cells were incubated with exosomes isolated from different groups, which were re-suspended in 5% FBS. After 20 min, the exosomes were replaced with normal growth medium. Apoptosis was detected by Annexin staining using a commercial kit (Annexin PE Apoptosis detection kit, BD Pharmingen, San Diego, CA), and analyzed on the FACS-Vantage SE flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Cortical neurons apoptosis were also detected by Hoechst staining. In brief, cortical neurons were grown on sterile cover glasses placed in the 6-well plates at a density of $1 \times 10^5$ cells/ml. After treated with exosomes, the cells were fixed in 4% paraformalehyde for 20 min at 4 °C. Fixed cells were washed three times with PBS, and stained with 10 mg/ml Hoechst 33342 staining solution according to the manufacture's instructions. Stained cell nuclei were observed under a laser scanning confocal microscope (Nikon MF30 led, Japan).

### 2.7. Statistical analysis

Data are expressed as mean ± SD from a minimum of three experiments. Student's t test was used to determine the significance of differences in multiple comparisons. A value of $P < 0.05$ was regarded as statistically significant.

## 3. Results

### 3.1. Electron microscopic analysis of exosome

The mouse microglia BV-2 cells were grown in RPMI1640 containing 5% FBS. Exosomes were isolated from the culture supernatant by ultrafiltration and ultracentrifugation. Electron microscopy revealed the presence of numerous secreted vesicles varying in size from 40 to 120 nm (Fig. 1). These vesicles were similar in shape and dimension to the previously described exosomes [20].



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