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The role of autophagy in the neurotoxicity of cationic PAMAM dendrimers

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ABSTRACT

Poly(amidoamine) (PAMAM) dendrimers, are among the most common classes of dendrimers that are intended for a wide range of biomedical applications and extensively investigated for brain-specific drug delivery, imaging and diagnosis. Unfortunately, neurotoxicity of PAMAM dendrimers, the underlying mechanism of which is poorly-elucidated, poses a far-reaching challenge to their practical applications. In this study, we reported that PAMAM dendrimers induced both cytotoxicity and autophagic flux in a panel of human glioma cell lines. Meanwhile, inhibition of autophagy significantly reversed cell death caused by PAMAM dendrimers, indicating the cytotoxic role of autophagy in neurotoxicity caused by PAMAM dendrimers. Akt/mTOR pathway was most likely to participate in initiation of PAMAM dendrimers-induced autophagy. Moreover, autophagy induced by PAMAM dendrimers might be partially mediated by intracellular ROS generation. Collectively, these data elucidated the critical role of autophagy in neurotoxicity associated with exposure to cationic PAMAM dendrimers *in vitro*, raising concerns about possible neurotoxic reaction caused by future clinical applications of PAMAM dendrimers and providing potential strategies to ameliorate toxic effects of PAMAM dendrimers.

1. Introduction

To date, with the rapid development of nanotechnology, considerable concerns have been raised about the potential hazardous effects of engineered nanomaterials on human health. In particular, toxicity of multifunctional nanomaterials specifically developed and engineered for biomedical applications, may restrict their further and extensive uses in drug delivery, imaging and diagnosis [1,2]. PAMAM dendrimers, a family of highly branched polymers that possess valuable inherent properties, such as monodispersity, multifunctionality and tunability, are potentially suitable and intensively investigated for biomedical applications in drug delivery, gene transfection and imaging [3–7]. Functionalized with surface primary amine groups, cationic PAMAM dendrimers display a positive charge and have been extensively documented to induce toxicity [8–19], such as initiate blood clot formation [11], disrupt platelet functions [12], promote acute lung injury [13] and cause toxic effects in the central nervous system [14], but the underlying molecular mechanism of these toxic effects is still indistinct. Regardless of the increasing significance of PAMAM dendrimers in brain-specific drug delivery and other biomedical applications, only a few experiments have been conducted to determine the neurotoxicity of the nanomaterials. Therefore, it is crucial to investigate potential neurotoxic effects of PAMAM dendrimers and especially the underlying molecular mechanisms, if they are to be practically exploited in the future.

It is generally recognized that dendrimers-induced cytotoxicity might be attributed to the interaction between positively charged dendrimers and oppositely charged cell membranes [20–22]. Recently, autophagy, a genetically regulated and evolutionarily conserved cellular process in which damaged or redundant components of cells are degraded, was speculated to be a universal cellular reaction in response to nanomaterials exposure [23]. Nanomaterials [24], such as carbon nanotubes [25], quantum dots [26], rare earth oxide nanocrystals [27,28], iron oxide, [29] titanium dioxide [30] and cerium dioxide nanoparticles [31,32], have been documented to induce autophagy in various cell lines. However, it remains controversial whether autophagy induced by nanoparticles is disruptive or not. While sometimes the elevated levels of autophagic activity may be an adaptive cellular response to

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degrade and clear nanomaterials, often the interaction between nanomaterials and cells is destructive, giving rise to excessive autophagy induction and severe morphological alteration, concomitant with cell death. Therefore, we hypothesized that the neurotoxicity associated with PAMAM dendrimers exposure was related to nanoparticles-induced autophagy and the application of autophagy inhibitors could attenuate neurotoxicity and thereby enhance security of PAMAM dendrimers for biomedical applications.

In this study, we aimed to characterize the molecular mechanisms of neurotoxicity induced by commercially available cationic PAMAM dendrimers and analyze the role of autophagy in neurotoxicity caused by the nanoparticles in a panel of human glioma cell lines.

2. Materials and methods

2.1. Preparation of PAMAM dendrimers G5

PAMAM dendrimers generation 5.0 with ethylenediamine core was purchased from Sigma–Aldrich (St. Louis, MO). After being air-dried under airflow of laminar flow cabinet for 36 h to remove methanol, PAMAM dendrimers G5 was dissolved in 0.01 M phosphate buffered saline (PBS) (pH7.4) to prepare a stock solution of 10 mg/mL [13].

2.2. Cell lines and culture conditions

The human glioblastoma cell lines U87MG, U251MG, U118 and A172 were obtained from Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). All human malignant glioma cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin and incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.3. Cell viability assay

Cell viability of human glioblastoma cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay. Briefly, cells harvested by trypsinization were seeded into 96-well plates and incubated overnight to allow for attachment, followed by treatments as indicated. After the cells were washed with PBS and incubated with MTT (0.5 mg/mL) for 4 h in a humidified incubator at 37 °C, the medium were carefully removed and the formed formazan crystals were thoroughly dissolved with DMSO (100 μL/well) by constant shaking. The absorbance at the wavelength of 570 nm was determined by a microplate reader. Cell viability was expressed as the ratio of absorbance of treated cells relative to that of untreated cells.

2.4. Morphological analysis

Differential interference contrast (DIC) module of inverted fluorescence microscope (Olympus IX71, Olympus, Japan) with a 10× magnification objective was employed to observe morphological alterations of cells subjected to treatments as indicated.

2.5. Confocal microscopy

2.5.1. Autophagosomes staining

[33] Cultured on glass bottom cell culture dishes (NEST Biotechnology, Jiangsu, China), human glioma cells were treated with or without PAMAM dendrimers G5 (100 μg/mL) for 12 h. Cells incubated with rapamycin (50 nM), an autophagy inducer, were served as positive controls. After indicated treatments, cells were stained with Cyto-ID Autophagy Detection Kit (ENZO Life Science, Farmingdale, NY, USA) following the manufacturer's instruction. In brief, after incubated with Cyto-ID Green dye and Hoechst 33342 for 30 min at 37 °C in a CO_2 incubator, cells were gently washed with 1× Assay Buffer and immediately observed by an inverted confocal microscope (Carl Zeiss LSM710, Carl Zeiss, Germany). Cyto-ID positive cells were counted manually using the ImageJ plugins cell counter.

2.5.2. Autophagosomes, lysosomes and autophagolysosomes staining

[34] After various experimental treatments, human glioma cells were co-stained with LysoTracker® Red (Invitrogen, San Diego, CA, USA), Cyto-ID Green Dye and Hoechst 33342 at 37 °C for 30 min. Then stained cells were gently washed twice with cell culture medium and subjected to confocal microscopy immediately.

2.6. Transmission electron microscopy

Human glioma cells were incubated with or without 100 μg/mL of PAMAM dendrimers G5 for 18 h. Cells collected by trypsinization were fixed with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) for 2 h at 4 °C and washed with 0.1 M cacodylate buffer containing 0.1% $CaCl_2$ for three times. Then the samples were post-fixed with 1% buffered osmium tetroxide (pH 7.4) for 30 min. After dehydration using graded ethanol, the samples were polymerized at 60 °C for 48 h, followed by being cut into ultrathin sections, stained with uranyl acetate and lead citrate, and finally examined by a JEM 1230 transmission electron microscope (JEOL USA, Inc.).

2.7. Immunoblot analysis

Cells collected in Eppendorf tubes were gently washed with cold PBS and homogenized in RIPA lysis buffer (Beyotime Biotechnology, Haimen, China) supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF) on ice for 30 min, followed by centrifugation (12000× *g*, 4 °C) for 10 min to clear the lysates. The concentrations of protein in total cell lysates were determined by bicinchoninic acid (BCA) method. Then cell lysates containing equivalent amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto polyvinylidene fluoride (PVDF) membranes. After being blocked with 5% (w/v) bovine serum albumin (BSA) in Tris-Buffered-Saline with Tween-20 (TBST) for 1 h at room temperature, the membranes were incubated overnight at 4 °C with primary antibodies and subsequently subjected to peroxidase-conjugated secondary antibodies for 2 h at room temperature. Finally, immunoreactive bands were developed by Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA). Densitometric values of immunoreactive bands were quantified by ImageJ 1.47v (National Institutes of Health, USA).

2.8. Measurement of intracellular ROS level

Reactive oxygen species assay kit (Beyotime Biotechnology, Haimen, China) was used to detect intracellular ROS generation in human glioma cell lines. Cells, seeded in 96-well black microplates, were incubated overnight to allow for attachment. After indicated experimental treatments for 2 h, cells were loaded with fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (10 μM) in serum-free medium for 30 min at 37 °C. Subsequently, cells were washed twice with serum-free medium and the DCF fluorescence intensity was measured using Tecan Infinite® 200 PRO microplate reader at Ex./Em. = 488/525 nm to quantify the levels of intracellular ROS.

2.9. Reagents and antibodies

3-Methyladenine (3-MA), chloroquine (CQ) and MTT were purchased from Sigma–Aldrich (St. Louis, MO, USA). Bafilomycin A1 (Baf A1) was obtained from Sangon Biotech (Shanghai, China). Purchased from Beyotime Biotechnology (Haimen, China) was N-acetyl-cysteine (NAC). The primary antibodies used for immunoblotting were as follows: anti-Phospho-mTOR (Ser2448), anti-Phospho-Akt (Ser 473), anti-Phospho-p70 S6 Kinase (Ser371), anti-LC3, anti-β-actin (Cell Signaling Technology, Danvers, MA, USA), anti-4E-BP1/2/3 Phospho (pT45) (Epitomics, Burlingame, CA, USA), anti-tubulin (Proteintech Group, Chicago, IL, USA). The secondary antibodies, including horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit immunoglobulin G (IgG), were obtained from MR Biotech (Shanghai, China).

2.10. Statistical analysis

The data were analyzed by GraphPad Prism 5 with one-way ANOVA or two-tailed Student's *t* test. All data were presented as means ± standard deviations (SD). *p* Values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Neurotoxicity and morphological alterations caused by PAMAM dendrimers

The viability of U87MG, U251MG, U118 and A172 cells in response to various concentrations of PAMAM dendrimers was determined by MTT cytotoxicity assay. After exposure to PAMAM dendrimers G5 for 24 h, the cell viability of human glioma cells was decreased in a dose-dependent manner within the tested dose range. Moreover, a pronounced reduction (up to ~60%) in cell viability was observed in human glioma cells treated with 100 μg/mL PAMAM dendrimers (Fig. 1A–D).

Similar results were further confirmed by morphology analysis with microscope. After incubation with increasing concentrations of PAMAM dendrimers, cells displayed obvious morphology changes, including reduced cell size, rounded shape, loss of neurites and decreased number of cells, while untreated cells appeared a classical morphology with a spindle and elongated shape (data not shown). In addition, more atrophic cells were observed when concentration of PAMAM dendrimers increased.

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