Dendrimer-Functionalized Multi-walled Carbon Nanotubes Exhibit Dual-Phase Regulation to Exposed Murine Embryonic Stem Cells

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Abstract

Herein we reported the effects of generation 5 polyamidoamine dendrimer-functionalized fluorescent multi-walled carbon nanotubes (dMNTs) on mice embryonic stem cell line CCE. The dMNTs were prepared and characterized, and incubated with murine embryonic stem cell CCE cells (ES) for 1 to 5 day. These ES cells were observed by fluorescent microscopy, and were analyzed by flow cytometer and MTT. Results showed that the dendrimer-functionalized fluorescent multi-walled carbon nanotubes were successfully synthesized, could enter into ES cells quickly, more than 20 µg mL\(^{-1}\) dose caused ES cells became smaller and smaller as the incubation time increased, and inhibited cell growth in dose- and time-dependent means, less than 5 µg mL\(^{-1}\) dose improves ES differentiation. These results demonstrate that dMNTs are toxic to ES cells at large dose, can induce ES cells’ differentiation at lower dose. The prepared dMNTs may be a good dual-phase regulation reagent to exposed ES cells, and have potential applications in research and development of ES cells.

Keywords: Embryonic stem cells, Dendrimer, Multi-walled carbon nanotubes, Proliferation, Differentiation


1. Introduction

Since embryonic stem cells (ES) were first isolated in the 1980s [1], the pluripotential nature of ES cells to differentiate into cell types of all three primary germ lineages display charming prospect for disease therapy [2-4]. ES cells’ study has been being made great progress. Although there exist many opportunities in study and development of stem cells today, there exist still great challenges in the technologies of generating progenitor cells with in-vivo reconstitution function and understanding the mechanisms of development and differentiation of ES cells, therefore it is very necessary to explore and develop new technologies.

Carbon nanotubes, as a class of stiff, stable and hollow nanomaterials with many unique properties such as mechanical, physical and chemical properties, have been being explored applications in medical chemistry and biomedical engineering [5,6]. Carbon nanotubes including both single-walled and multi-walled carbon nanotubes, have been actively functionalized with different biological molecules and investigated as possible multifunctional biological transporters [7,8]. Previous reports show that single-walled carbon nanotubes can take antisense oligonucleotides [9], plasmid DNA [10], siRNA [11], and peptides [12] across cell membrane, and also can be used as near-infrared agents for selective cancer cell imaging and therapy [13,14].

Dendrimers are a novel special class of organic molecules: they can take different functional groups through a series of chemical modifications, and their interior cavities can serve as storage areas for a lot of genes or drugs [15,16]. Dendrimers may be a good nonviral delivery vector because it has the advantages of safety, simplicity of use, and ease of mass production compared with viral vectors that are inherently risky.
for clinical use [17,18]. Polyamidoamine (PAMAM) dendrimer-modified magnetic nanoparticles can markedly enhance the delivery efficiency for antisense oligonucleotides [19], dendrimer-modified single walled carbon nanotubes can reduce cytotoxicity and enhance the cellular uptake of the nanoparticles [20-24], and can release slowly those absorbed genes for two weeks.

Here we fully use advantages of multi-walled carbon nanotubes and polyamidoamine dendrimer, firstly investigated the effects of generation 5 dendrimer-functionalized multi-walled carbon nanotubes on mouse ES CCE cells with the aim of developing new method of regulating development and differentiation of ES cells based on nanomaterials.

2. Experimental Section

2.1 Materials source

Ethylenediamine and methylacrylate were purchased from Aldrich Chemical Company. Multi-walled carbon nanotubes(MCNTs) were obtained from Shenzhen Nanopost Company (Shenzhen, China). Murine ES CCE cell line was kept in consolidated research institute for advanced science and medical care, Waseday university. RPMI 1640 medium containing 10% fetal calf serum was from Gibco Company. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was obtained from Dojin Laboratories (Kumamoto, Japan). Rabbit antimouse monoclonal antibody against fibronectin, β-actin antibody, and horseradish peroxidase(HRP)-conjugated goat anti-rabbit secondary antibody were from Invitrogen Co. Enhanced chemiluminescence kits were from Amersham Company (Germany).

2.2 Synthesis of dendrimer-functionalized multi-walled carbon nanotubes

MNTs were added to 60% aqueous nitric acids. The mixture was placed in an ultrasonic bath for 60 min and then stirred for 24 h while being boiled under reflux. The mixture was then vacuum-filtered through a 0.22 µm Millipore polycarbonate membrane and subsequently washed with distilled water until the pH of the filtrate was ca. 7. The filtered solid was dried under vacuum for 24 h at 70°C, yielding MWNT-COOH. The dried MWNT-COOH was suspended in SOCl₂ and stirred for 48 h at 70°C. The solution was filtered, washed with anhydrous THF, and dried under vacuum at room temperature for 48 h, generating MWNT-COCI. The dried MWNT-COCI was mixed with ethylenediamine at a ratio of 1:2 and stirred for 48 h at 80°C. The resulting solid was separated by vacuum-filtration using a 0.22 µm Millipore polycarbonate membrane filter and subsequently washed with water, generating MWNT-NH₂. 50 mg dried CNT-NH₂ was dispersed in 20 ml of 20% methylacrylate aqueous solution. The suspension was immersed in a sonication water bath at 25°C for 3 h. The particles were then washed with water. After washing, 20ml of a 1:1 methanol-ethylenediamine solution was then added to the complex, and the mixture was allowed to proceed under the same conditions. Stepwise growth using methylacrylate and ethylenediamine was repeated until the No.5 generation of dendrimer-modified MNTs was achieved. The CdTe nanocrystals with COOH group mixed with dMNTs, the resultant fluorescent dMNTs were washed 3 times with 25 ml water for usage [25,26].

2.3 Characterization of dendrimer-modified multi-walled carbon nanotubes

High-resolution transmission electron microscopy (HR-TEM, Hitachi H-700H, Hitachi, Japan) was used to confirm the size of the carbon nanotubes and observe the dendrimer-coating layer on the MNTs. Zeta potential was measured with Zetasizer 2000 instruments (Malvern Co., England). Atomic force microscopy (AFM) imaging was performed by a Nanoscope III (Digital Instruments/Veeco Metrology Group, United States). AFM images were
obtained in the tapping mode with standard Si/N tips.

2.4 ES Cells’ culture and MTT analysis

ES CCE cells were cultured in RPMI 1640, containing $1 \times 10^5$ mU mL$^{-1}$ of penicillin and 0.1 mg mL$^{-1}$ of streptomycin supplemented with 10% (v/v) FCS, at 37 °C in a humidified 5% CO$_2$ and 95% air atmosphere. The mediums were exchanged once every 2 days. The cells were observed by contrast optical microscopy. An MTT (tetrazolium salt) assay was conducted to evaluate the effects of dMNTs on ES CCE cells. The inhibition curve of cell proliferation can be drawn [27,28].

2.5 Morphologic observation of ES cells incubated with dMNTs

All ES cells were seeded into 6 well-chambered cover slides for ~24 h in an RPMI 1640 cell growth medium. 40 µg ml$^{-1}$ dMNTs were added into the medium and co-cultured with ES cells at 37°C for 24 h in 5% CO$_2$ atmosphere. Then the cells were fixed with 2.5% glutaraldehyde in PBS at 4°C for 20 min. Finally, the cells were observed through fluorescent microscopy (Nikon 2000, Japan), and the cellular images were recorded.

3. Results and Discussion

3.1 Characterization of dendrimer-functionalized multi-walled carbon nanotubes

As shown in Fig. 1a, synthesized fluorescent multi-walled carbon nanotubes own green fluorescent signal in culture medium, similar to previous report [29]. As shown in Fig. 1b and 1c, dendrimer coating layer existed on the surface of multi-walled carbon nanotubes. As shown in Fig. 2, generation5 dendrimer-functionalized fluorescent multi-walled carbon nanotubes exhibited stronger positive charge than unmodified multi-walled carbon nanotubes. These results demonstrate that dendrimer-functionalized fluorescent carbon nanotubes were successfully synthesized.

3.2 Effects of dMNTs on the adhesion and proliferation of ES cells

As shown in Fig. 3, as the incubation day increased, the ES cells gradually floated, became smaller and smaller, which showed that dMNTs can decrease the adhesive ability of ES cells, may induce apoptosis of ES cells. The culture medium, similar to previous report [29]. As shown in Fig. 1b and 1c, dendrimer coating layer existed on the surface of multi-walled carbon nanotubes. As shown in Fig. 2, generation5 dendrimer-functionalized fluorescent multi-walled carbon nanotubes exhibited stronger positive charge than unmodified multi-walled carbon nanotubes. These results demonstrate that dendrimer-functionalized fluorescent carbon nanotubes were successfully synthesized.

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ES cell survival curves showed that dMNTs inhibited proliferation of ES cells in dose-and time-dependent means.

As shown in Fig. 4, as the incubation day of ES cells increased, compared with the control, the expression levels of fibronectin protein in ES cells became lower and lower, which showed that ES cells’ adhesive ability decreased gradually as the incubation time increased.

3.3 Effects of dMNTs on the differentiations of ES cells

As shown in Fig. 5 (left image), fluorescent multi-walled carbon nanotubes located win ES cells, which showed that dMNTs can enter into ES cells. As shown in Fig. 5 (middle and right images), part ES cells were differentiated into long tree-branched cells. When the ES cells were incubated with 5 µg ml\(^{-1}\) dMNTs in medium for No.5 day, the differentiated cells could be observed, which showed that low dose of dMNTs can improve differentiation of ES cells. We also used flow cytometer to confirm the ES cells’ differentiation (data not shown), showing that low dose of FCNTs can improve the differentiation of ES cells.

Although carbon nanotubes have been broadly investigated their application [30], up to date, how to deliver siRNA or protein into ES cells is still a great challengeable task. Our primary results showed that polyamidoamine dendrimer-functionalized fluorescent carbon nanotubes could enter into ES cells, may be a good nonviral gene or drug delivery system for ES cells, similar to our previous report [6,7]. Our results also showed that large dose of dMNTs (> 20 µg ml\(^{-1}\)) can inhibit proliferation of ES cells in dose-and time-dependent means, make ES cells become smaller and smaller, part ES cells appeared apoptosis, low dose of dMNTs (<5 µg ml\(^{-1}\)) can improve the differentiation of ES cells. The dMNTs maybe low toxic to ES cells within the scope of special doses. Regarding the dendrimer-functionalized fluorescent multi-walled carbon nanotubes’ toxic mechanism, some reports gave different explanation [31-33], however, we consider that it is closely associated with surface positive charges of dMNTs, which can be used to combine with genes or drugs, the dendrimer itself maybe no toxic to ES cells, which is also showed that ES cell membrane should be able to endure positive charges at some degree. However, the positive charge increases and overdue the limitation of cell membrane tolerance, which will result in cell membrane destruction, and inducing cell apoptosis or death. Under the scope of tolerance of cell membrane, positive charge of dendrimer can induce the nanoscale hole formation in the surface of ES cells by interaction with lipid protein on the surface of ES cell membrane, which will provide the rapid pathway for entrance of dendrimer-modified MCNTs into ES cells, therefore, dendrimer-modified MCNTs own high efficiency of entering into ES cells, which also indirectly showed that dendrimer-functionalized fluorescent multi-walled carbon nanotubes may be a good gene or drug delivery system for ES cells, and have potential applications in ES cells’ imaging and functional research.

4. Conclusion

The dendrimer-functionalized fluorescent multi-walled carbon nanotubes were successfully prepared, and exhibit dual-phase regulation to ES CCE cells, lose dose of FMCNTs can improve the proliferation and differentiation of ES cells, high dose of FCNTs can inhibit the growth of ES cells, which may be a good regulation reagent to control growth and differentiation of ES cells, and have potential applications in ES cells’ imaging and functional research.
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