ELSEVIER

Nanomedicine: Nanotechnology, Biology, and Medicine
xx (2014) xxx – xxx

nanomedicine
Nanotechnology, Biology, and Medicine

nanomedjournal.com

Toxicity of carboxylated carbon nanotubes in endothelial cells is attenuated by stimulation of the autophagic flux with the release of nanomaterial in autophagic vesicles

Martina Orecna, PhD[a,1], Silvia H. De Paoli, PhD[a,1], Olga Janouskova, PhD[b,c], Tseday Z. Tegegn, BS[a], Marcela Filipova, MS[b], John E. Bonevich, PhD[d], Karel Holada, PhD[b], Jan Simak, PhD[a,*]

[a]*Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD, USA*
[b]*Institute of Immunology and Microbiology, 1st Faculty of Medicine, Charles University in Prague, Prague, Czech Republic*
[c]*Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Prague Czech Republic*
[d]*National Institute of Standards and Technology, Gaithersburg, MD, USA*

Received 4 November 2013; accepted 12 February 2014

Abstract

Carbon nanotubes (CNTs) exhibit a number of unique properties that make them attractive for various nanomedicine applications including their intravascular use. Therefore, the vascular toxicity of CNTs is a critical safety concern and methods of CNTs toxicity modulation are of great interest. Here, we report that carboxylated multiwalled carbon nanotubes (MWCNTs) induce a decrease in viability of cultured human umbilical vein endothelial cells (HUVECs) associated with the profound accumulation of autophagosomes. This autophagosome accumulation was mTOR kinase independent and was caused by blockade of the autophagic flux rather than by activation of autophagy. Stimulation of the autophagic flux with 1 nmol/L bafilomycin A1 attenuated the cytotoxicity of carboxylated MWCNTs in HUVECs and was associated with the extracellular release of the nanomaterial in autophagic microvesicles. Thus, pharmacological stimulation of the autophagic flux may represent a new method of cytoprotection against toxic effects of nanomaterials.
Published by Elsevier Inc.

Key words: Carbon nanotubes; Autophagy; Bafilomycin A1; Exocytosis; Microvesicles

Abbreviations: BafA1, bafilomycin A1; CCK-8, cell counting kit-8; CNT, carbon nanotubes; CTRL, vehicle treated control; CQ, chloroquine; DTS, dense tubular system; ER, endoplasmatic reticulum; FESEM, field emission scanning electron microscopy; GFP, green fluorescence protein; GRASP, Golgi reassembly and stacking protein; HUVEC, human umbilical vein endothelial cells; ICP-MS, inductively coupled plasma mass spectrometry; LC3, microtubule-associated light chain 3; LDH, lactate dehydrogenase; LSCM, laser scanning confocal microscopy; 3-MA, 3-methyladenine; mTOR, mammalian target of rapamycin; MV, microvesicle; MVB, multivesicular bodies; MWCNT, multiwalled carbon nanotube; M60, multiwalled carbon nanotube with average outer diameter 60 nm from SES Research; M60COOH, carboxylated M60; PI, propidium iodide; RFP, red fluorescence protein; SD, standard deviation; WB, Western blotting.

Disclaimer: The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy. Opinions expressed in this paper are the authors' and do not necessarily reflect the policies and views of the National Institute of Standards and Technology (NIST), or affiliated venues. Certain commercial equipment, instruments, or materials are identified in this paper only to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the NIST, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

Funding: This project was supported in part by an appointment to the Research Participation Program at the Center for Biologics Evaluation and Research administered by the Oak Ridge Institute for Science and Education through and interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration. In addition, the study was supported in part by the Grant LH12014 from the Ministry of Education, Youth and Sports of Czech Republic. M.F., K.H. and O.J. were supported by projects of Charles University in Prague: SVV260026, PRVOUK-P24/LF1/3 and UNCE 204022, respectively.

Disclosures: The authors have no disclosures.

*Corresponding author at: Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD 20852-1448, USA.
E-mail address: jan.simak@fda.hhs.gov (J. Simak).

[1] These authors contributed equally to this publication.

http://dx.doi.org/10.1016/j.nano.2014.02.001
1549-9634/Published by Elsevier Inc.

Carbon nanotubes (CNTs), one of the key emerging types of nanomaterials, exhibit a broad range of potential applications in nanomedicine.[1–3] Because of their unique mechanical, thermal and electronic properties, CNTs are very attractive candidates for use in diagnostic biosensors, drug delivery nanosystems, imaging nanoprobes for intravascular use and other devices that come into direct contact with blood.[4–6] The toxicological profiling of CNTs, which has included a number of controversial studies, has attracted great attention in recent years. It has been shown that inhaled CNTs are able to cross the epithelial barrier[7] to reach the circulation and cause thrombus formation.[8,9] Therefore, evaluation of the blood and vascular toxicity of CNTs is a critical safety issue. The cytotoxicity of CNTs has been extensively studied,[10] including examining the effects of CNTs on vascular endothelial cells.[11–19] Surface carboxylation of CNTs is a common strategy for increasing their solubility in aqueous solvents for biological applications. However, the mechanism underlying the effects of carboxylated CNTs on vascular endothelial cells has not been studied thus far.

The purposes of our study were to investigate cytotoxicity of pristine and carboxylated multiwalled CNTs (MWCNTs) in cultured human umbilical vein endothelial cells (HUVECs) and to elucidate a mechanism of the effect of carboxylated MWCNTs on HUVEC autophagy. Here, we show that in contrast to their pristine counterparts, carboxylated multiwalled MWCNTs induced autophagosome accumulation in cultured HUVECs. Autophagy (macroautophagy, "self-eating") is a degradation pathway in which cytoplasmic content is engulfed and degraded by the lysosome.[20,21] Autophagy is essential for cells to break down their own components; however, when dysregulated either by inhibition or activation, it leads to cell injury and death.[22] The classical pathway of autophagy activation involves inhibition of the protein kinase mTOR (mammalian target of rapamycin). It has been reported that some nanomaterials can induce autophagy by modulating mTOR activity.[22] In contrast, the autophagosome accumulation observed in the present study, induced by carboxylated MWCNTs, was mTOR independent and was caused by blockade of the autophagic flux, rather than by activation of autophagy. Interestingly, stimulation of the autophagic flux with 1 nmol/L bafilomycin A1 (BafA1) attenuated the cytotoxicity of carboxylated MWCNTs in HUVECs and was associated with the endothelial release of CNT-containing autophagic vesicles.

Methods

Detailed description of experimental procedures is included in Supplementary Materials. Water-soluble carboxylated MWCNTs M60COOH were prepared from the pristine MWCNTs with an average diameter of 60 nm (M60) obtained from SES Research (Houston, TX).[23,24] HUVECs (second passage of pooled primary cells) from Lonza (Walkersville, MD), were cultured as published previously.[25] The CCK-8 (Cell Counting Kit-8, Dojindo Molecular Technologies, Inc., Rockville, MD) assay was used to determine the effect of different nanomaterials on cell viability.[11] Effect of different nanomaterials on the lactate dehydrogenase (LDH) release by HUVECs was detected by a LDH Cytotoxicity Detection Kit (Takara Bio, Inc., Otsu, Japan).[11]

The flow cytometry Annexin V/propidium iodide (PI) apoptosis detection kit (BD Biosciences, San Diego, CA) and assay of the activation marker CD54 were performed as described previously.[26]

Western blotting analysis of HUVECs was performed using anti LC3B rabbit polyclonal antibody (1:1000; Invitrogen, Carlsbad, CA), anti p70S6 kinase (49D7) rabbit polyclonal antibody (1:1000; Cell Signaling, Danvers, MA), anti p-p70S6 kinase (T389) (1A5) rabbit polyclonal antibody (1:1000; Cell Signaling), or anti SQSTM1/p62 mouse monoclonal antibody (1:1000; Abcam, Cambridge, MA). β-Tubulin (1:5000; Abcam) was used as a loading control.[27,28]

Flow cytometric analysis of HUVEC-released microvesicles (MVs) was performed in HUVECs tranduced with the Premo™ Autophagy Sensor LC3B-GFP BacMam 2.0 kit (Invitrogen) and treated with Alexa Fluor® 647 conjugated M60COOH with or without 1 nmol/L BafA1. Counts of LC3-GFP$^+$MVs and Alexa Fluor 647-M60COOH$^+$ MVs in media were evaluated using double-fluorescence plots. MV counts per microliter of media were calculated.[29]

Laser scanning confocal microscopy (LSCM) was used for detection of the autophagosome marker LC3B in cell MVs and HUVECs transduced with Premo™ Autophagy Sensor LC3B-GFP and treated with M60, M60COOH or Alexa Fluor® 555 or Alexa Fluor® 647 conjugated M60COOH with or without 1 nmol/L BafA1. Additional staining for actin (Phaloidin 555, Invitrogen) and nuclei (TO-PRO, Invitrogen) was performed.

The ultrastructural analysis of the CNTs-HUVECs interaction was performed by the field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM). Real-time PCR analysis of 84 genes involved in autophagy, apoptosis or necrosis was performed using The Human Cell Death Pathway Finder RT^2 Profiler PCR Array (SA Biosciences, Qiagen).[30]

Statistical analysis

Data are presented as means + standard deviation (SD). If not specified otherwise, the results were calculated from three independent experiments. The data were plotted and analyzed using GraphPad Prism 5.0 Software (GraphPad Software, Inc. San Diego, CA). One-way ANOVA was used; P values <0.05 were considered significant.

Results

We studied the effects of pristine and carboxylated MWCNTs on cultured endothelial cells. We selected pristine MWCNTs with an average diameter of 60 nm (M60) obtained from SES Research[23,24] due to their relatively high purity, as determined via inductively coupled plasma mass spectrometry (ICP-MS) and transmission electron microscopy (TEM) (Table S1, Figure S1 in the Supplementary Materials). Carboxylated MWCNTs (M60COOH) were prepared by refluxing M60 MWCNTs in a concentrated mixture of H_2SO_4 and HNO_3, followed by washing and dialysis in water. The structures of the M60 and M60COOH aggregates were determined by electron microscopy. Pristine M60 in aqueous solutions is highly hydrophobic, which tends to lead to the formation of bundles and larger agglomerates.