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Commercial single-walled carbon nanotubes effects in fibrinolysis of human umbilical vein endothelial cells

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ABSTRACT

Recent studies have demonstrated that carbon nanotubes (CNTs) induce platelet aggregation, endothelial dysfunction and vascular thrombosis. However, there is little information on the effects of CNTs on fibrinolysis. We investigated the role of pristine-commercial single-walled carbon nanotubes (SWCNTs) with <3% Co content in fibrinolysis and their contribution to the induction of pro-thrombotic processes in human vein endothelial cells (HUVEC). SWCNTs alone produced concentration-dependent oxidation, as measured by a dithiothreitol oxidation assay. Internalized SWCNTs were located in HUVEC treated with 25 μg/ml using transmission electron microscopy, whereas treatment with 50 μg/ml compromised cell viability, and oxidative stress increased significantly at 5 μg/ml. The study showed that in HUVEC treated with 25 μg SWCNT/ml, fibrinolysis-related gene expression and protein levels had increased by 3–12 h after treatment (*serpine-1*: 13-fold; *PLAT*: 11-fold and *PLAU*: 2-fold), but only the PAI-1 protein was increased (1.5-fold), whereas tissue and urokinase plasminogen activator proteins (tPA and uPA, respectively) tended to decrease. In summary, pristine SWCNTs treatment resulted in evident HUVEC damage caused by cell fiber contact, internalization, and oxidative stress due to contaminant metals. The generation of endothelial dysfunction, as shown by the altered expression of genes and proteins involved in fibrinolysis, suggest that SWCNTs display pro-thrombotic effects.

1. Introduction

At present, nanomaterials have become a valuable raw material used in industry for numerous products with special properties. Carbon nanotubes (CNTs) are a case in point as, since their discovery in 1991 (Iijima, 1991), they have attracted great scientific research and technological attention due to their simple chemical composition, extraordinary properties and versatile applications. However, the human risk of exposure to nanomaterials has not yet been fully characterized (for a review, see Rodriguez-Yanez et al. (2013)). Exposure to CNTs may occur in an occupationally context or derived from the possible use as medical devices. Also, due to the increasing production and use of CNTs, also will increase their release to the environment during their production process. For these reasons, respiratory and vascular system represent a portal of entry of CNTs. Nevertheless, several research groups have studied the biodistribution pathway using different animal models. It is clear that CNT-toxicokinetics depends on the shape, physicochemical properties, impurities and agglomeration of pristine CNTs. These characteristics also determine the CNT-deposition in different regions of the respiratory tract when they are inhaled. To date, there are only a few studies that explain the toxicokinetic

Abbreviations: CNTs, carbon nanotubes; SWCNTs, single-walled carbon nanotubes; MWCNTs, multi-walled carbon nanotubes; HUVEC, human umbilical vein endothelial cells; ECs, endothelial cells; PAs, plasminogen activators; tPA, tissue-plasminogen activator; uPA, urokinase-plasminogen activator; PAI-1, plasminogen activator-1; KKS, kallikrein-kinin system; KLK1, tissue kallikrein; FBS, fetal bovine serum; TNF-α, tumor necrosis factor-alpha; DTT, dithiothreitol; PI, propidium iodide; C-TEM, conventional transmission electron microscope; HR-TEM, high-resolution transmission electron microscope; H_2DCFDA, 2,7-dichlorofluorescein diacetate; ROS, reactive oxygen species; cDNA, complementary DNA; PCR, real-time polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; FS, fibrinolysis system.

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of CNTs, but the most of them have been focused use either as intra-tracheal instillation or intravenous injection, and explain the effects on respiratory and systemic blood circulation. It is possible that after their deposition, CNTs are able to translocate to extrapulmonary sites (Bussy et al., 2013; Ali-Boucetta and Kostarelos, 2013). In this context, Erdely et al. (2013) reported a relationship between pulmonary deposition and systemic circulation, where the CNTs may induce both pulmonary and systemic effects characterized by a blood gene and protein expression pattern. Once CNTs enter the general circulation, they may interact with blood cells, plasma proteins and endothelial cells (ECs).

ECs form a single cell layer called the endothelium, which is localized at the luminal face of the blood vessels and plays a key physiological role in the regulation of fibrinolysis mechanisms through the expression of pro-thrombotic and anti-thrombotic factors in close relationship with smooth muscle cells and the extracellular matrix (Baudin et al., 2007). Human umbilical vein endothelial cells (HUVEC) represent a good model commonly used for physiological, pharmacological and toxicological investigations, such as blood coagulation, macromolecule transport, angiogenesis and fibrinolysis (Bachetti and Morbidelli, 2000) and also for the study of endothelial cell functions due to the fact that they are both anatomically and physiologically representative of arterial blood vessels. Thus, moreover, represents a first approach to the search for mechanisms to explain cellular alterations due to diseases or to the presence of toxic agents.

Fibrinolysis is a complex process with many steps and the participation of numerous molecules, e.g., (a) plasminogen, a pro-enzyme that is converted into plasmin, the active enzyme, depending on the presence of plasminogen activators (PAs), such as tissue-plasminogen activator (tPA) and urokinase-plasminogen activator (uPA), and their inhibitors (PAIs), especially plasminogen activator-1 (PAI-1); (b) the kallikrein-kinin system (KKS), particularly tissue kallikrein (KLK1), which promotes fibrinolysis by stimulating the release of tPA from ECs (Brown et al., 2000; Bhoola et al., 1992).

In vivo, exposure to CNTs results in endothelial dysfunction with neutrophil adhesion to the endothelial monolayer through increases in the expression of adhesion molecules via nuclear NF-κB/P65 translocation, as well as oxidative stress (Zhiqing et al., 2010), the induction of platelet aggregation and an increase in thrombus formation, leading to a decrease in the occlusion time in small mesenteric arteries, which increases vascular thrombosis (Radomski et al., 2005; Bihari et al., 2010). In addition, CNTs affect platelets, which are essential to the coagulation process, promoting their activation *in vitro* leading to their aggregation and viability loss. This process is associated with Ca^{2+} influx and its depletion from intracellular stores (Lacerda et al., 2011; Semberova et al., 2009; Meng et al., 2012), increases in both P-selectin expression and the number of platelet-granulocyte complexes (Bihari et al., 2010), and an up-regulation of glycoprotein integrin receptor GPIIb/IIIa, all of which are crucial for platelet aggregation (Radomski et al., 2005). Additionally, CNTs cause different degrees of red blood cell damage, leading to earlier fibrin formation that might significantly increase the hardness of the clots (Meng et al., 2012).

In spite of the increasing experimental data on the pulmonary effects of CNTs, at present, there are few studies focused on the effects of CNTs on the vascular endothelium, especially toward the coagulation system.

Due to the lack of information about the mechanism by which CNTs may affect fibrinolysis, we investigated the effects of commercial and pristine SWCNTs on primary cultures of human umbilical vein endothelial cells (HUVEC) *in vitro* by assessing the expression of genes/proteins associated with plasminogen activation (*Serpine-1*/PAI-1, *PLAT*/tPA, *PLAU*/uPA), vascular homeostasis (KLK1) and endothelial dysfunction, because ECs might interact with this nanomaterial. In addition, the intrinsic SWCNTs oxidative potential, their cell internalization and their capacity to produced oxidative stress were also studied.

2. Materials and methods

2.1. Carbon nanotubes suspension in culture media

A single-walled carbon nanotubes (SWCNTs) stock was purchased as dry powder from Nanostructured & Amorphous Materials, Inc. (Houston, TX); the physicochemical characterization of SWCNTs is shown in Table 1. The powder sample was dissolved in sterile deionized (DI) H_2O using a biosafety cabinet class III (Esco Technologies, Inc., Singapore, Sin) at a concentration of 4 mg/ml. The stock solution was prepared as previously described (Wang et al., 2010a). The mixture was suspended by sonication at 90 W for 15 min (Branson, Danbury, CT) and used as a stock solution. An appropriate amount of the SWCNT stock solution was added to the culture media to obtain the desired final concentration. The diluted suspensions were vortexed for 15 s, then sonicated for 15 min and vortexed again for 15 s. Here, we used fetal bovine serum (FBS) (Gibco-Life Technologies) as a SWCNT-dispersing agent, and it was added to the culture media before the addition of SWCNTs at a 1% v/v final concentration. Thereafter, we characterized the SWCNT-suspension by assessing its stability index through monitoring the sedimentation kinetics of the suspension's absorbance at 550 nm for different lengths of time in a UV–VIS spectrophotometer (Infinite M200, Tecan Austria GmbH, Austria). An aliquot (1 ml) of the SWCNT-suspension containing 50 μg/ml was prepared with or without FBS, and the absorbance readings were taken at 1 hour-time intervals for 24 h (data not shown).

2.2. Dithiothreitol assay and oxidative activity

The intrinsic redox activity was adapted for SWCNTs from the assay based on the ability of particulate matter components to act as electron transfer agents between dithiothreitol (DTT), as the electron source, and oxygen, as the electron acceptor (Kumagai et al., 2002). The remaining thiol content was allowed to react with DTNB, generating 5-mercapto-2-nitrobenzoic acid, and measured at 412 nm in a spectrophotometer. Briefly, the SWCNT-suspensions (0.01–50 μg corresponding to the following theoretical molarities: 2.94×10^{-15} – 6.32×10^{-16} to 1.47×10^{-11} – 3.16×10^{-12}, as considering the lower and higher SWCNTs dimension limits; http://www.nanointegris.com/en/hipco) were incubated at 37 °C with 0.5 M PBS, pH 7.4, DI H_2O and 1 mM DTT (Sigma–Aldrich) for 0–45 min. Subsequently, 10% trichloroacetic acid (Sigma–Aldrich) was added to an aliquot of the incubation mixture to stop the reaction. Next, an aliquot of the last mixture was dissolved with Tris buffer, pH 8.9, with 20 mM EDTA and 10 mM DTNB (Sigma–Aldrich) solution and

Table 1
Physicochemical characterization of SWCNTs.

Property	Characterization
Purity	95% CNT, 90% SWCNT
Average outside diameter	1–2 nm
Inside diameter	0.8–1.6 nm
Length	1–3 μm
Impurities/contaminants	
Transition metals	Co < 3%
Other elements	Al < 0.1%, Cl < 0.5%, S < 0.3%

Stock#: 1246YJS. Manufacturing method: Catalytic CVD. Analysis Method: Energy Dispersive X-ray Spectroscopy, as reported by the supplier.