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Determinants of the thrombogenic potential of multiwalled carbon nanotubes

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ABSTRACT

Multiwalled carbon nanotubes (MWCNTs) are cylindrical tubes of graphitic carbon with unique physical and electrical properties. MWCNTs are being explored for a variety of diagnostic and therapeutic applications. Successful biomedical application of MWCNTs will require compatibility with normal circulatory components, including constituents of the hemostatic cascades. In this manuscript, we compare the thrombotic activity of MWCNTs *in vitro* and *in vivo*. We also assess the influence of functionalization of MWCNTs on thrombotic activity. *In vitro,* MWCNT activate the intrinsic pathway of coagulation as measured by activated partial thromboplastin time (aPTT) assays. Functionalization by amidation or carboxylation enhances this procoagulant activity. Mechanistic studies demonstrate that MWCNTs enhance propagation of the intrinsic pathway via a non-classical mechanism strongly dependent on factor IX. MWCNTs preferentially associate with factor IXa and may provide a platform that enhances its enzymatic activity. In addition to their effects on the coagulation cascade, MWCNTs activate platelets *in vitro*, with amidated MWCNTs exhibiting greater platelet activation than carboxylated or pristine MWCNTs. However, contrasting trends are obtained *in vivo*, where functionalization tends to diminish rather than enhance procoagulant activity. Thus, following systemic injection of MWCNTs in mice, pristine MWCNTs decreased platelet counts, increased vWF, and increased D-dimers. In contrast, carboxylated MWCNTS exhibited little procoagulant tendency *in vivo*, eliciting only a mild and transient decrease in platelets. Amidated MWCNTs elicited no statistically significant change in platelet count. Further, neither carboxylated nor amidated MWCNTs increased vWF or D-dimers in mouse plasma. We conclude that the procoagulant tendencies of MWCNTs observed *in vitro* are not necessarily recapitulated *in vivo*. Further, functionalization can markedly attenuate the procoagulant activity of MWCNTs *in vivo*. This work will inform the rational development of biocompatible MWCNTs for systemic delivery.

1. Introduction

Carbon nanotubes (CNTs) consist of sheets of graphitic carbon formed into single-walled or multiwalled tubes (termed SWCNTs or MWCNTs, respectively). The unique combination of electrical, thermal and spectroscopic properties of these materials offers new opportunities for advances in the detection, monitoring and treatment of diseases including cancer [1]. For example, our group has shown that MWCNTs directly injected into tumors are highly effective agents for anti-tumor thermal therapy, resulting in long term remission of ~80% of tumors implanted in mice [2]. Moreover, CNTs can be taken up by cells, allowing them to act as efficient carriers of therapeutic drugs and nucleic acids [3,4]. The ability to incorporate multiple diagnostic and therapeutic functions into a single particle makes carbon nanotubes well-suited for further biomedical development [5,6].

The toxicity profile of MWCNTs will be a critical factor in determining their clinical utility. Examination of the toxicological profile of CNTs has largely focused on adverse effects induced by environmental exposure to CNTs, such as those created by inhalation or during manufacture. Such studies have raised concerns

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regarding the possibility that long (10–20 μm) CNTs induce asbestos-like reactions [7], or that inhalation of CNTs causes granulomatous pneumonia, oxidative stress, and acute inflammatory and cytokine responses [8–10]. However, many uses of CNTs as nanomedicines, including implementation as anti-cancer drugs, will necessitate systemic administration through intravenous and other routes, where alternative toxicities might be observed. Because of the well-documented ability of intravascular particulates to induce thrombosis, induction of undesired thrombotic events represents a particularly critical potential toxicity [11–14].

Many systemic components contribute to thrombosis, including coagulation factors of the intrinsic and extrinsic coagulation cascade, platelets, and endothelial cells. The effect of CNTs on some of these components has been studied. For example, pristine nanotubes without surfactant coatings have been reported to activate platelets *in vitro* and accelerate the formation of thrombi in the microvasculature of rodents following artificial induction of the hemostatic cascade [12,13,15–18]. However, the role of specific components of the hemostatic system, such as proteins of the coagulation cascade, as well as how MWCNTs affect the interplay among these components, remains largely unexplored. Further, CNTs used in systemic applications are almost invariably dispersed with surfactants; effects of these therapeutically relevant combinations of functionalized MWCNTs and surfactants on coagulation have not been investigated.

Typical modifications for enhanced CNT biocompatibility include covalent functionalization of the nanotube exterior with carboxyl groups (reviewed in [19]) and/or "wrapping" in long-chain surfactants (reviewed in [20]). Antibodies or other targeting moieties have also been linked to CNTs to both aid in their dispersion and promote their accumulation in tumor tissue [21,22]. It has been suggested that covalent functionalization can improve the overall toxicity profile of carbon nanotubes by enhancing their clearance [23]. In the present study, we assessed the potential thrombogenic effects of functionalized MWCNTs *in vitro* and *in vivo*.

2. Materials and methods

Complete experimental details are provided in SI Materials and Methods.

2.1. Materials

Pristine (PD15L1-5, Lot number: 40209) carboxyl (PD15L1-5–COOH, lot number: 60809) and amide functionalized (PD15L1-5–NH_2, lot number: 60809) multiwalled carbon nanotubes were purchased from NanoLab (Waltham, MA). MWCNTs were suspended in sterile saline with 1% (wt/wt) pluronic F127 (Sigma) or DSPE-PEG (Avanti Polar Lipids) through probe tip sonication (Branson). Uncoated MWCNT suspensions were prepared using sterile saline as the solvent. All samples were autoclaved prior to use.

2.2. Platelet activation

Aliquots of PRP were incubated with increasing concentrations (10, 50 or 100 μg/mL) of either uncoated, pluronic F127 or DSPE-PEG coated MWCNTs (pristine, carboxylated or amidated) (see *Materials*) for 30 min at room temperature. Samples were then labeled with 20 μL of FITC-conjugated CD62P (555523) (BD Biosciences) for 15 min at room temperature. Platelets were then fixed in 1× formaldehyde and diluted to 1 mL final sample volume in PBS. Samples were analyzed on a FACSCaliber flow cytometer and data analyzed using CellQuest Pro software (BD Biosciences). Platelets incubated with 23 μM ADP served as the positive control while a sample fixed with 1× formaldehyde prior to ADP stimulation constituted the negative control.

2.3. Platelet aggregation

Aliquots of platelet rich plasma were incubated with 100 μg per mL pluronic F127 or DSPE-PEG_{5000} coated MWCNTs (pristine, carboxylated or amidated) (see Materials) for 30 min at room temperature. Samples were then labeled with 20 μL of FITC-conjugated CD62p (555523) (BD Biosciences) for 15 min at room temperature. Platelets were then fixed in formaldehyde and diluted to 1 mL final sample volume in PBS. Samples were centrifuged at 800×*g* for 15 min and washed with fresh PBS three times to remove plasma proteins and unbound antibody. 200 μL of washed and resuspended platelets were placed onto poly-L-lysine coated cover slips and allowed to adhere overnight at 4 °C in a moistened chamber. Cover slips were then washed three times in fresh PBS and mounted on slides using 1:1 glycerol/PBS mounting medium. Platelets were imaged using an Axiovert 100M (Zeiss) confocal fluorescence microscope.

2.4. Activated partial thromboplastin time (aPTT)

Asolectin stock was prepared by homogenizing asolectin (Sigma Aldrich) at 3.8% (w/w) in physiologic saline. Asolectin was further diluted 1:35 in Owren's Veronal buffer (Dade Behring) immediately prior to use. Kaolin stock was prepared by suspending kaolin in physiologic saline at a final concentration of 20 mg/mL or 100 μg/mL.

For the aPTT assay, 50 μL of activator (either kaolin or MWCNTs) was mixed with 50 μL of diluted asolectin stock and added to 100 μL of pooled normal plasma (PNP) (George King Biomedical) in a 300 μL reaction cup and allowed to incubate for 2 min. The sample was then placed in a fibrometer (BBL Fibrosystems) and activated with 100 μL of 0.025 M calcium chloride solution. The clotting reaction was allowed to proceed to completion and the time recorded.

2.5. Nanotube binding of factor IX and factor IXa

Purified human factor IX and factor IXa (Haematologic Technologies Inc.) were individually reconstituted in TBS buffer containing 2.5 mM Ca^{2+} and 1.4 mg/mL BSA to a final concentration of 100 nM. For the experiment described in Figure S3 calcium was not added to the buffer for the samples described as "calcium-free". The indicated nanotube preparations in either pluronic F127 or DSPE-PEG were then diluted in these solutions to final concentrations of 100 or 250 μg/mL and incubated for 1 h at room temperature. Following incubation, samples were centrifuged at 14,000×*g* in a Beckman microcentrifuge for 30 min to pellet the nanotubes and any bound protein. Control samples containing factor IX or factor IXa in buffer but no nanotubes were similarly centrifuged. Aliquots from the supernatant of each sample were withdrawn and proteins resolved by SDS-PAGE using 10% polyacrylamide gels. Gels were transferred to nitrocellulose and probed with a monoclonal mouse anti-human factor IX antibody (AHIX-5041) from Haematologic Technologies Inc. Membranes were developed by enhanced chemiluminescence method and imaged in a LAS 3000 (Fuji Medical Systems). Relative band intensities were determined using the Image J software package.

2.6. Mouse model

To establish baseline platelet counts 25 μl of blood was drawn from the tail vein by venipuncture and collected in a citrated pipette. Blood was immediately diluted in 5 ml of Isoton II buffer (Beckman Coulter) then centrifuged at 200×*g* for 8 min to prepare platelet rich plasma (PRP). 100 μl of PRP was further diluted in 10 ml of Isoton II and platelets were counted using a Beckman Coulter Z2 particle count and size analyzer.

Mice were anesthetized by isoflurane inhalation, weighed, and their tails were dipped into 50 °C water to dilate the veins. A total of 250 μg of nanotubes in a 250 μl volume was administered by tail vein injection to 4-5 mice per nanotube/surfactant combination (See *Materials*). As a control, normal saline containing 1% pluronic F-127 was mixed with an equal volume of normal saline containing 1% DSPE-PEG and 250 μl of the admixture was injected into the tail vein of 4 mice. Blood was again collected from the tail vein 3 h, 24 h, and 48 h after nanotube injection, and platelets were counted as described above. Additionally, mice were monitored for signs of physical distress.

All mice were sacrificed 48 h after nanotube injection and approximately 300–500 μl of blood was collected from the inferior vena cava and placed into citrated tubes. Blood was centrifuged at 5000×*g* in a Beckman microcentrifuge and plasma was collected and immediately frozen at −80 °C for further analysis for D-dimer and mVWF.

2.7. Mouse d-dimer and vWF ELISA

Mouse plasma was diluted 1:5 in phosphate buffered saline and D-dimer (E0506Mu) or mVWF (E0833Mu) levels were quantified by ELISA using a commercial assay kit (Uscn Life Science Inc.) according to the manufacturer's instructions.

2.8. Histology and analysis

Mice were euthanized by CO_2 asphyxiation and sections of kidney, liver, lung, heart and spleen were preserved in 10% neutral buffered formalin. The tissues were routinely processed for histology and stained with hematoxylin (HE) and phosphotungstic acid hematoxylin (PTAH). Slides were examined for evidence of MWCNT-mediated tissue damage by a board certified anatomic veterinary pathologist (NDK).